Review of *Nucleophaga* (a primitive, 'cryptomycotan' genus): Summary of named and unnamed species, with discussion of contemporary and historical observations

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ABSTRACT

Genus *Nucleophaga* is a parasite of the nucleus of various protozoa, i.e., a number of amoebae (free-living and parasitic forms), types of flagellates, and allegedly certain ciliates. *Nucleophaga* was, since its original description (Dangeard, 1895), considered to be fungal (a Chytridiomycete). Within Chytridiomycetes, the genus was included in family Olpidiaceae (Sparrow, 1960; Karling, 1977) along with a similar genus, *Sphaerita* (occurring in the cytoplasm rather than the nucleus), and with *Olpidium* (some species of which have been more recently determined, cf. James et al. 2006, to have membership in groups other than Chytridiomycota). Recent work (e.g., Corsaro et al., 2014, 2016) has indicated, however, that closer relationships of *Nucleophaga* are with Cryptomycota ('Rozellomycota' of some), and perhaps even more so with Microsporidia (Bass et al., 2018). In any case, only two species of *Nucleophaga* (*N. amoebae* and *N. terricolae*) have been examined in molecular and electron-microscopic studies. And, no single publication has yet fully accounted for all named species of the genus and a number of putative species remaining unnamed. Part of the reason for the relatively poorly known status of potential taxa of *Nucleophaga*, among mycologists, is that much early publication on the genus was in protozoological (rather than mycological, or botanical) literature--related to the fact that early observers of *Nucleophaga* were often interested in protozoa as primary subjects of investigation. The purpose of our study is to review literature (including that of protozoology) and bring named species of *Nucleophaga* together, and, importantly, provide discussion of each. A further goal is to assemble an annotated list of potential (unnamed) additional species. We exclude forms once considered to possibly belong to *Nucleophaga*, but which, in all probability, do not. We hope our collation of information will encourage additional finds (and molecular and electron microscopic investigations) of the genus--now understood, along with related genera, to be significant in understanding early fungal/protist (holomycotan) evolution.
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As part of a growing interest in what has come to be known as the 'holomycotan assemblage' of organisms (Fungi and protistan relatives), increasing attention has been directed to groups--such as the Cryptomycota ("Rozellomycota" of some authors), the Chytridiomycota, the Microsporidia, the Aphelidea and the Choanozoa--which may provide further insights into connections between, and evolution of, primitive fungi (e.g., the Rozellida) and primitive animals (e.g., types of 'protozoa' such as Nuclearids). Increasing focus (especially molecular study) is being given to those organisms, among primitive 'holomycotans,' that are poorly understood or the systematic placement of which has remained uncertain--in an attempt to fill in gaps in present knowledge and provide missing pieces of the puzzle of early eukaryotic diversification (e.g., Corsaro et al., 2018). There are many such poorly known, primitive organisms (of various types), a number being parasitic; most are in need of taxonomic summaries, where available information (even if scattered or incomplete) is compiled in a given study. Our present summary focuses on one genus, *Nucleophaga*--described by Dangeard (1895)--in part because of the recent attention two of its species have received (Corsaro et al., 2014, 2016), but also because its existence (even if not known by name, for a time) has been 'documented' over many years (e.g., see Kirby's, 1941, mention of work by Carter, 1863 and Greeff, 1866). Following Dangeard's (1895) formal description of

Nucleophaga, there has been a long subsequent history of observation of the genus (particularly in literature of protozoology), as will be evident in our presentation.

Obviously, the recovery of living examples of poorly known forms of any of the above groups (Chytridiomycota, Cryptomycota, Microsporidia, etc.) or the discovery of new forms--for molecular study--is of high value in evolutionary investigations. A recent example of this type of study (molecular analysis of obscure holomycotans) is that of Corsaro et al. (2014) who indicated finding, and provided sequence information for, *Nucleophaga amoebae* (a nuclear parasite of a type of amoeba)--originally described, as noted, by Dangeard (1895). This study of Corsaro et al. (2014) was founded in part on the morphological (electron-microscopic) work of Michel et al. (2009a). These investigations underscored the importance of such updated studies. *Nucleophaga*, traditionally considered a member of the Chytridiomycota (family Olpidiaceae), was determined to constitute a distinct lineage, related to types of Microsporidia and also (if somewhat more distantly) to genus *Rozella*. *Nucleophaga amoebae* appeared to represent a 'missing link,' or evolutionary 'intermediate step,' within the Cryptomycota.

The stated "rediscovery" (see Corsaro et al., 2014, title page) of the parasite, *Nucleophaga amoebae*, was in the free-living amoeba, *Thecamoeba quadrilineata*--found in a "moss meshwork" (Michel et al., 2009a). Corsaro et al. (2016) soon described a second species of *Nucleophaga*, parasitizing another free-living amoeba (*Thecamoeba terricola*), found on sycamore bark. This second *Nucleophaga* (Corsaro et al., 2016) was determined to be morphologically and genetically similar to, but nonetheless distinct (especially based on molecular analysis) from, *N. amoebae* (cf. Michel et al., 2012; Corsaro et al., 2016). Corsaro et al. (2016) named this second *Nucleophaga*, *N. terricolae*. These two *Nucleophaga* species (*N. amoebae* and *N. terricolae*) were considered (Corsaro et al., 2016) together to constitute a distinct, 'sister' lineage within a general group of microsporidian organisms.

The two *Nucleophaga* species above, *N. amoebae* ('recovered;' Corsaro et al., 2014) and *N. terricolae* (newly described; Corsaro et al., 2016)--along with one additional species, *N. ranarum*, described years before (Lavier, 1935b) from amoebae found in Amphibians--were, until recently, the only three *Nucleophaga* species (species names) listed in Index Fungorum (IF, an updated listing of fungal names). Presently, five names are listed in IF (including the addition of *N. intestinalis* (Brug, 1926) and *N. peranemae* (Hollande & Balsac, 1941); however, a sixth species, *N. hypertrophica* (Epstein, 1922), is not included (as of this writing). In any case, additional potential species of *Nucleophaga* have been described (though not named), a subject of our investigation. Additional forms of *Nucleophaga*--or related organisms--are important to phylogenetic investigation, since such may place near the base of fungal radiation (Bass et al., 2018), and relate to evolution of primitive protozoa (Corsaro et al., 2018).

A number of reviews (several early reviews are summarized by Kirby, 1941) of potential hosts of *Nucleophaga*, and related organisms, are helpful in understanding possible taxa of *Nucleophaga*. This is especially true since *Nucleophaga*--though from the start (Dangeard, 1895) considered to be essentially 'fungal' (for many years viewed as 'chytridiaceous') in character--occurs in (parasitizes) a number of different amoebae (free-living and parasitic forms), at least two major groups of flagellates (Kirby, 1941), and "ciliates of various kinds" (Calkins, 1933, p. 386)--a statement echoed by Sleigh (1989, p. 277). Sleigh mentioned "rumen ciliates from mammals" as possible hosts [seemingly for *Nucleophaga*], a suggestion, however, in need of verification. In fact, as regards the Ciliata as a whole, further clarification is required as to precisely *which* genera/species are parasitized by *Nucleophaga*; i.e., specific examples seem elusive. Calkins (1909, his fig. 118A) did, though, produce a convincing illustration of a probable *Nucleophaga* in the macronucleus of *Paramecium aurelia*. Nonetheless, in various 'reviews' of occurrence in the literature (e.g., Sassuchin, 1934; Kirby, 1941; Sleigh, 1989), it can often be difficult to be sure whether nuclear (*Nucleophaga*) or cytoplasmic (*Sphaerita*) parasites are being discussed--see, for example, Sassuchin's (1934, p. 216) discussion of "parasitism due to fungi."

In any case, a substantial portion of the information about *Nucleophaga* is to be found in (often older) protozoological, rather than mycological (or botanical), literature (see comments in this regard by Sparrow, 1960, p. 125; and Dick, 2001, p. 407). Helpful reviews (or studies with a related review constituent) include those of Pénard (1905), Mercier (1907), Mattes (1924), Brug (1926), Sassuchin (1934), Brumpt and Lavier (1935), Lavier (1935b), Hollande and Balsac (1941) and Kirby (1941). The strong protozoological component to the literature of *Nucleophaga* accounts in part for why some named species, and additional potential species, of the genus were not always noted in mycological listings, e.g., *Index Fungorum* (IF). A more recent (mycological) review--of *Sphaerita*, *Pseudosphaerita*, *Morella* and *Nucleophaga*--was published by Karling (1972); however, the focus of his paper was to the concepts and potential distinctions of these putative (possibly related) genera, rather than, necessarily, determination of taxa within these genera. Karling's (1977) later coverage of *Nucleophaga* was more sub-generically useful, discussing and illustrating several species. No names for *Nucleophaga* species were added in Longcore's (1996) enumeration. Dick (2001) recognized three species of *Nucleophaga* (accounting for a fourth name, as an alleged synonym)--a listing differing from that of *Index Fungorum*, in that Dick recognized *N. hypertrophica* Epstein (1922).

Hopefully, our listings (below) will provide additional historical and current context of *Nucleophaga*. With the possible exception of *N. amoebae* and *N. terricolae* (listed first, List One)--these being carefully molecularly investigated by Corsaro et al. (2014, 2016, respectively), and well-studied morphologically by Michel et al. (2009a, 2012, respectively)--any of the remaining 'species' (Lists One and Two) should be reinvestigated, since these taxa have not been subjects of molecular or electron microscopic analysis. Such putative taxa, and other primitive fungal parasites of protozoa discovered, would be worth the effort of collecting and studying for possible new insights and connections they may reveal concerning early fungal/protist evolution. We exclude only those forms (List Three)--though at one point suggested to represent *Nucleophaga*--that present scrutiny reveals do not belong in the genus.

TAXONOMIC LISTINGS

We present below three listings: List One is six named, potential species of *Nucleophaga*. List Two is seven, unnamed, but likewise potential taxa of the genus. Presentations are given with attempted fidelity to authors' statements. These lists constitute a more comprehensive, annotated survey of possible species than found elsewhere. List Three is excluded forms. All figures mentioned in text refer to figs. *in* publications of *other* authors, with the exception that our **Figs. 1-19** (these illustrating species from **List One** only) are here redrawn (from the original sources indicated in our three figure-legends).

LIST ONE: The six, named species of *Nucleophaga*. Because it is not certain any of these taxa are identical, we treat them as potentially distinct. Species 1, 2 and 6 parasitize free-living protozoa; whereas, 3, 4 and 5 are 'hyperparasites,' parasitizing protozoa which are potentially parasitic. See our Figs. 1-19 (species #2, morphologically similar to #1 under the light microscope, is not separately illustrated).

1. *Nucleophaga amoebae* Dangeard (1895). The genus *Nucleophaga* was established by Dangeard (1895), as well as the original species, *N. amoebae*. It was discovered by Dangeard in the free-living *Amoeba*, *A. verrucosa*, which Pénard (1905) considered a form of *A. proteus* (see also Kirby, 1941). Useful illustrations of *Nucleophaga amoebae* are Dangeard's (1895) figs. 3 and 4--his fig. 4A (p. 210), for example, seems to be an illustration cited in broad treatments of Chytridiomycetes such as Sparrow (1960, p. 124, fig. 12A) and Karling (1977, p. 31, pl. 12, fig. 9). A distinct sporangial membrane is interpreted to be present in this *Nucleophaga* (see Dangeard's fig. 4D; and see our Discussion, 2nd paragraph); Dangeard's figs. 4B,C,E,F nicely illustrate that more than one [*N. amoebae*] parasite can be present in a given host-nucleus. As for taxonomic evaluation of the host, there is an indication in Hall (1953) that *Amoeba verrucosa* should in fact be *Thecamoeba verrucosa* (see also below).

Corsaro et al. (2014) indicated recouping *Nucleophaga amoebae* (Dangeard, 1895) in the free-living amoeba, *Thecamoeba quadrilineata*--see also Michel et al. (2009a). Micrographs of the *Nucleophaga* in Michel et al. and Corsaro et al. do bear similarity to drawings provided by Dangeard (mentioned above). However, it should also be mentioned here that the amoeba species examined by Michel et al. and Corsaro et al., *Thecamoeba quadrilineata*, is apparently *not* the same species of amoeba examined by Dangeard (viz. *Amoeba verrucosa* = *Thecamoeba verrucosa*; cf. Bovee, 1985); in fact, *Thecamoeba quadrilineata* may be more similar to *T. sphaeronucleolus* than it is to *T. verrucosa* (see the taxonomic account of Bovee, pp. 172-173). Regardless of the question of the absolute certainty of its identity to the organism originally observed by Dangeard (1895), the *Nucleophaga* studied by Corsaro et al. (2014), in *Thecamoeba quadrilineata*, is indeed the first *Nucleophaga* species to be genetically sequenced--a study of unquestioned value in determining the true relationships of genus *Nucleophaga*.

2. *Nucleophaga terricolae* Corsaro et al. (2016). Described by Corsaro et al. (2016), and related to *N. amoebae* (see above), this is the only *other* species of *Nucleophaga* that has been sequenced (and is, thus, presented here next). It was isolated from *Thecamoeba terricola*, a free-living amoeba found on sycamore bark (Michel et al., 2012). Under the light microscope, *N. terricolae* is very similar to *N. amoebae* (and is not here separately illustrated). Corsaro et al. (2016) determined *Nucleophaga terricolae* to be 94.5 % genetically identical (18S rDNA) with *N. amoebae*, forming (together) a distinct clade within various microsporidian organisms--all within a broadly defined (Corsaro et al., 2016) Rozellomycota (= Cryptomycota) including *Rozella*. Though having an appearance and developmental cycle similar to *N. amoebae*, *N. terricolae* showed (under TEM) minor morphological differences--including slightly more polymorphic and amoebic trophont-stages and, additionally, slightly larger, later ('spore') stages--see Corsaro et al. (2016, p. 3005). As for hosts, the amoeba, *Thecamoeba terricola*, in which *N. terricolae* occurs, is a distinct species from the *Thecamoeba* in which Corsaro et al. (2014) studied *N. amoebae* (see Bovee, 1985, who, by the way, noted that "*Nucleophaga*" parasitizes *Thecamoeba terricola*).

At an early date, Mattes (1924) described an organism which he named "*Sphaerita nucleophaga,*" later discussed by Karling (1972). Mattes (1924) indicated this organism occurred within the nucleus of the following three *Amoeba* species: "*A. sphaeronucleolus*," "*A. vespertilio*," and (less often) "*A. terricola*." This "*Sphaerita*" of Mattes is, in all probability, *Nucleophaga*--not *Sphaerita*, which would occur in the cytoplasm rather than the nucleus. It is questionable, though, that the apparent *Nucleophaga* organisms in the three different amoeba species are the same; for example, the *Nucleophaga* (cf. Mattes, plate 19, figs. 17-24), as we refer to it here, in *Amoeba sphraeronucleolus* pushed the remains of the nucleolus to the periphery of the nucleus, flattening it as a residual cap (of relatively limited extend); whereas, the putative *Nucleophaga* in *Amoeba terricola* (his plate 20, figs. 32,33,34), apparently, 'marginalized' the chromatin material as a thin layer, spread more or less uniformly around the inner face of the nuclear membrane. Be this a consistent difference or not, one may wonder if Mattes (1924; see his plate 20, e.g., figs. 32, 34) might have been observing, in *Amoeba* (*Thecamoeba*) *terricola*, a species of *Nucleophaga* similar to that described by Corsaro et al. (2016), viz. *N. terricolae*? The tightly packed spore-mass of the nuclear parasite shown in the morphological work of Michel et al. (2012; see fig. 1b, p. 38) on *Thecamoeba terricola*--a basis for the study of Corsaro et al., 2016, in which *Nucleophaga terricolae* was described--is indeed similar to that in fig. 34 (plate 20) of Mattes (1924); the main difference *apparently* is a narrow, 'empty' zone around the spore mass, evident, if not labeled, in fig. 1b, Michel et al. (2012); this zone possibly represents peripheral 'space' where depleted chromatin material, or scattered remnants of the nucleolus (Michel et al., fig. 5b), existed--as such became marginalized, prior to destruction by nucleophagy. In addition to Mattes (1924), even earlier, Pénard (1905, p. 196) may have observed what eventually came to be called *Nucleophaga terricolae* in *Amoeba* (*Thecamoeba*) *terricola*, although Pénard seemingly assumed (due to a generally similar appearance) it was the same as the parasite initially described by Dangeard (1895) in '*Amoeba verrucosa*,' viz. *Nucleophaga amoebae*.

3. *Nucleophaga hypertrophica* Epstein (1922). Epstein, writing in Russian (providing a German summary), described the species, *N. hypertrophica*, found in the human parasitic amoeba, *Endolimax nana* (occurring in the colon and caecum; cf. Manwell, 1961). *Endolimax nana* also occurs in other mammals such as swine and monkeys; *E. nana* is similar in size to, but morphologically distinguishable (particularly in the cyst stage) from, the amoeba discussed under #4 below. As a parasite of a parasite, *Nucleophaga hypertrophica* would, by definition (see Manwell), be a 'hyperparasite.' The name *hypertrophica*, however, indicates enlargement (hypertrophy) of the amoeba nucleus as a result of parasitism by *Nucleophaga.* Figures 1,3,6,7,9,14 in Epstein (1922) clearly indicate this parasite is *Nucleophaga*; a spore of this organism may infect the nucleolus (Epstein, 1922, his fig. 3), subsequently dividing internally (fig. 6 of Epstein, 1922) in a central position and displacing chromatin material outward (this pattern may constitute a difference from *N. intestinalis*, discussed below). Nöller (1921) may have observed what was *N. hypertrophica* a year earlier (see Wenyon, 1926, p. 253; Kirby, 1941, p. 1046), though seemingly not naming it. Epstein (1922) did not appear to base his study and descriptive information on Nöller (1921), Nöller's work not being cited by Epstein. In any case, see discussions concerning Nöller and Epstein in Lavier (1935b) and also Kirby (1941). In Epstein's (1922) fig. 9, there is a hint of a 'clear zone' surrounding the *Nucleophaga* spores--discussed under #4, below.

4. *Nucleophaga intestinalis* Brug (1926). This organism was described as a parasite of a human intestinal amoeba that would eventually be named *Iodamoeba buetschlii* (which has been spelled *I. bütschlii,* cf. Manwell, 1961); it was formerly named *Endolimax williamsi*, cf. Levine (1973). This amoeba is the most common amoeba in swine (Levine, 1973). Brug (1926) studied *Nucleophaga intestinalis* from a prepared slide, a number of examples being observed. This nuclear parasite (aggregation of coccoid spores) was observed to be surrounded by a 'clear zone' or 'space,' rather than a sporangial membrane; Brug (p. 468) thus spoke of the "nakedness" of this *Nucleophaga*, and considered it distinct. In [*apparently*] lacking a sporangial membrane, there is resemblance to #3, above, *Nucleophaga hypertrophica* (Epstein, 1922; see Kirby, 1941, p. 1046). Brug (1926), though, seemed unaware of Epstein's work. Brumpt and Lavier (1935) considered Brug's species (*intestinalis*) a synonym of Epstein's species (*hypertrophica*), as did Dick (2001). Nonetheless, Karling (1977) appeared to recognize Brug's species. An illustration of what is probably *N. intestinalis* is in Kudo (1966, p. 1074, fig. 387b). It is our belief that, since the host amoebae originally studied by Epstein (1922) and Brug (1926) were distinct genera, there is insufficient basis to be certain the respective *Nucleophaga* species infecting them were the same--regardless of subsequent statements (Brumpt and Lavier, 1935; Epstein, 1935; Kirby, 1941)--especially in the absence of molecular data. These two hyperparasites could prove to be different species, regardless of similar appearance; *N. intestinalis* appears to push and flatten the nucleolus outward, rather than directly infecting it within (as in *N. hypertrophica*); in either case, the nucleolus ultimately disappears (due to probable nucleophagy).

5. *Nucleophaga ranarum* Lavier (1935b). This species of *Nucleophaga* was found parasitizing *Entamoeba ranarum* occurring in intestines of tadpoles of the European Midwife Toad, *Alytes obstetricans* (see Lavier, 1935b). As a parasite of a parasite, this *Nucleophaga* would be considered (as in #3 and 4 above) a 'hyperparasite.' *Nucleophaga ranarum* is noted by Index *Fungorum* (IF), but not by Dick (2001). Because the *N. ranarum* life cycle seemed a combination of the two types of development said to occur in *Nucleophaga* -- see though Kirby, 1941, who thought the *Nucleophaga* described by Lavier (1935b) could represent a 'type one' development; but, contrastingly, see our Discussion, second paragraph -- Lavier (1935b) considered this species distinct, providing a species name, *N. ranarum*. This species seems unusual in that, at a very young stage, a plasmodial-like structure appears to engulf the nucleolus and adjacent portions of the nucleus (Lavier's, 1935b, fig. 6, pl. 10). Though first mentioned (but not named to species) in Lavier (1935a), Lavier (1935b) published the more complete account of this species later that year--both publications in French, and in parasitological/protozoological literature. If the publication (1935b) is deemed 'protozoological,' one *might assume* the organism described would have been named in accordance with the zoological code of nomenclature. However, since *Nucleophaga* was, from its inception (Dangeard, 1895), considered a Chytridiomycete (therefore fungal in nature)--and since

Lavier (1935b) also considered it a chytrid--it should have been named in accordance with the botanical code pertaining at the time (fungi being regarded, *for nomenclatural purposes*, as 'plants'). This might seem a relatively moot point, except that the botanical code, by then, *required* a diagnosis *in Latin* (beginning Jan. 1, 1935, i.e., the year in which *N. ranarum* was subsequently described). Since no Latin diagnosis was given by Lavier (1935a or 1935b), a diagnosis (in Latin *or* English; cf. McNeill et al., 2012) would need to be provided if it were desired to formally validate this name.

6. *Nucleophaga peranemae* Hollande & Balsac (1941). This species was discovered as a nuclear parasite in *Peranema trichophorum* (a free-living, heterotrophic, colorless euglenoid organism). Since this species was found in a protozoan (euglenoid flagellate) other than an amoeba, and since it was observed to form a (minutely spiny) resting spore or cyst (see pp. 38, 41, and fig. 14 in Hollande & Balsac)--a resting spore not otherwise reported in *Nucleophaga*--this parasite was considered to be a new species of *Nucleophaga*. Hollande and Balsac placed value (with which we agree), in determining species, on the host occupied, this host being quite different from those previously reported for *Nucleophaga*. The development of this species appears to correspond to 'type-one' listed in Kirby (1941)--see 2nd paragraph of our Discussion--although this species was not covered by Kirby (1941) since Kirby's was a contemporaneous publication. Hollande and Balsac's (1941) illustrations (e.g., their fig. 6) of the sporangium indicate that the parasite develops as a plasmodium with a surrounding membrane (although this is not necessarily clear from their text discussion). It is perhaps worth noting that, contrary to Hollande and Balsac's claim, theirs is not the first mention of occurrence of *Nucleophaga* in a flagellate (i.e., in protozoa other than amoebae). Kirby (1940), in another publication, noted finding *Nucleophaga* in a different type of flagellate--*Caduceia theobromae*--a 'Devescovinid' flagellate (see #6, List Two), such flagellates occurring in the digestive tract of termites. *Nucleophaga peranemae* was recognized by Dick (2001, p. 407); as noted by Dick, though, validation of this name would require supplying a proper diagnosis.

LIST TWO: Probable additional species of *Nucleophaga* (not named), given here as "*Nucleophaga* sp." Since there have been many observations of *Nucleophaga* over time, our listing will surely prove incomplete; further possible species are mentioned in the last paragraph of our Discussion.

1. *Nucleophaga* sp. (see Tyzzer, 1920). Tyzzer (1920) described, as new, a relatively small species of amoeba--*Pygolimax gregariniformis*--occurring in the caecum of young turkeys (and apparently also chickens). In nuclei of some of these amoebae, he found (by means of stained slides) small, more or less coccoid inclusions that had pushed the 'karyosome' (nucleolus) to one side; he also found that the nucleus often became somewhat hypertrophied. In some instances, the nuclear membrane ruptured--the remains of the nucleolus frequently still visible for a time after this occurred. Tyzzer mentioned *Nucleophaga* as a possibility for the organism (nuclear parasite) he found, and noted similarity to Dangeard's species, *N. amoebae*. However, Tyzzer also noted that the coccoid bodies he observed in the protozoan material from turkeys were somewhat smaller (0.4 to 0.8 µm) and not uniformly spheroidal, as compared with those in Dangeard's material (where the coccoid bodies were from 1 to as much as 2.5 µm, and consistently spheroidal). Tyzzer did not apply a species name to his organism, though obviously wondering if it were different (based on size and morphology, and occurring as it did in a distinct host). Certain statements by Tyzzer, and his figures 12-14 (plate 22), appear to confirm this organism as a *Nucleophaga*, possibly a different species--in any case, an organism well worth reinvestigating.

2. *Nucleophaga* sp. (see Kirby, 1927). In describing a new species of amoeba (*Endamoeba disparata*)--occurring in the intestine of a termite (*Mirotermes hispaniolae*) found on Barro Colorado Island, the Canal Zone, Panama--Kirby (1927) noted and illustrated (his figs. 40-45, plate 24) a *Nucleophaga* (not named to species) infecting the nucleus of the amoeba; numerous instances of this parasitism were observed. Although *Nucleophaga* organisms were found in several species of amoebae (at this location) by Kirby, the one here discussed is of particular interest, not only for its occurrence in a newly described amoeba, but because of a seemingly unique mode of development. In various *Nucleophaga* species, the

nucleolus (chromatin material of the host) is either devoured from within or pushed to the perimeter and flattened against the host nuclear membrane in the process of its utilization. However, in the *Nucleophaga* in *E. disparata*, the coccoid bodies of the parasite develop first around the periphery of the host nucleus (inside, of course, the nuclear membrane), enclosing a 'central zone' in which the mass of chromatin (nucleolar) material of the host is for a time 'contained' (see Kirby's, 1927, figs. 40, 43; and Kirby's, 1941, figs. 218B-E). Eventually, though, the *Nucleophaga* will 'fill-in' the nucleus, host nucleolar material being destroyed. Kirby (1932) also observed (with sparse comment) a few examples of a *Nucleophaga* seen in *Endamoeba beamonti*--occurring in another genus of termites (*Amitermes*) found at Barro Colorado Island; the distinction of this additional *Nucleophaga* remains to be determined.

3. *Nucleophaga* sp. (see Sassuchin, 1934). In examining an intestinal amoeba (*Entamoeba citelli*) occurring in *Citellus pygmaeus* (the 'steppe suslik,' a type of ground-squirrel) from Russia, Sassuchin (1934) found 'hyperparasites' in this amoeba--one of which occurred in (sometimes eventually filling) the nucleus, potentially causing significant hypertrophy. Sassuchin determined this nuclear parasite to be *Nucleophaga*, evidenced by his figs. 5 and 6. Sassuchin did not provide a species name. Kirby (1941) considered 'Sassuchin's *Nucleophaga*' to possibly represented an example of the so-called 'type-two' development of *Nucleophaga* (i.e., no multinucleate, membrane-bounded, plasmodial or sporangial structure evident). Sparrow (1960) simply noted Sassuchin's reference as an example of the numerous mentionings of *Nucleophaga* in protozoological literature. Puzzling, is that Dick (2001) listed Sassuchin's (1934) reference (thus, presumably the *Nucleophaga* that Sassuchin was observing) under both *Nucleophaga hypertrophica* (therefore also *N. intestinalis*, considered a synonym by Dick) and *N. peranemae*--both species seeming unlikely placements (for Sassuchin's organism) because of the quite different hosts involved (particularly in the case of *N. peranemae*). It is more likely that Sassuchin's *Nucleophaga* would prove to be a distinct species, if it could be examined again.

4. *Nucleophaga* sp. (see Kirby, 1941; Karling, 1971). As discussed by Karling (1977), Kirby (1941) noted a probable *Nucleophaga* in *Pseudospora volvocis* (*Pseudospora* being a parasitic, or sometimes 'scavenger,' amoeba that may also have a flagellated stage) which had invaded the alga, *Volvox*. As further noted (Kirby, 1941; Karling, 1977), this interpretation (of spore-like bodies of a probable *Nucleophaga* parasitizing nuclei of *Pseudospora*) was based on earlier observations (Robertson, 1905) in which these 'bodies' or 'spheres' were then interpreted as uniflagellate gametes of *Pseudospora* that were *somehow* forming *within* the *Pseudospora* nucleus (not a likely scenario). Roskin (1927), however, clarified the situation to a considerable extent (including by his own observations) in pointing out that the 'gametes' (sphere-like structures) seen by Robertson (1905) corresponded generally to structures (small bodies seen in nuclei of hosts) assignable to a Chytridiaceous fungus (a good determination at the time)--not unlike structures observed and so interpreted by other authors in that general time-frame (e.g., Epstein, 1922). Figures 15 and 19 in Roberston (1905) indeed appear to represent *Nucleophaga*, pressing the nucleolus against the nuclear membrane. Her figure 20a shows motile cells, each with a subapically to laterally (i.e., generally anteriorly) attached, somewhat curved, trailing flagellum; her figure 20b illustrates two of these motile cells fused (the resultant cell being biflagellate--a zygote?). So, how to interpret all of this? First, the organism, as concluded by Kirby (1941) and Karling (1977), *probably is* a *Nucleophaga* (occurring, unquestionably, in the *Pseudospora* nucleus). It is probably not truly a chytrid, as would now be understood--chytrid zoospores (and gametes) being posteriorly flagellate (i.e., a precise posterior flagellar attachment). There are though some forms, such as *Olpidiomorpha*--that *may* prove to belong to the Cryptomycota--in which a subapical (at least somewhat anterior) attachment of a trailing flagellum occurs; this is probably also true for species of *Sphaerita* (cf. Sparrow, 1960). So, in the case of what Robertson (1905) observed, and Roskin (1927) and Kirby (1941) more correctly interpreted, one could make a case that the motile structures seen by Robertson were possibly gametes [zoospores?], not of *Pseudospora*, but of a 'cryptomycotaceous fungus'--*Nucleophaga* being a good possibility. So, why is this of significance? It is so because, if correctly interpreted by Kirby (1941) and Karling (1977), this is the only example of motile cells found in *Nucleophaga*, and the only instance of potential sexual

reproduction in the genus. It would be especially interesting to study this organism again, and confirm the existence of motility in a genus typically disseminating only by non-motile spores (endospores).

5. *Nucleophaga* sp. (see Kirby, 1941). *Trichonympha* is in Family Trichonymphidae (see Lee, 1985b), a group of 'hypermastigote' flagellates related to Family Devescovinidae, and more broadly to Trichomonads (see Hall, 1953; Dyer, 1990; Blackwell and Powell, 2006; see also our mention of Devescovinids under #6, List One--and, below, in this listing). Kirby (1941, pp. 1059-1063) noted--in *Trichonympha* found in termites, *Procryptotermes,* studied in Madagascar and in Java--the occurrence of a parasite with *Nucleophaga*-like development. In this case, chromatin material of the host-nucleolus became outwardly displaced (by development of the *Nucleophaga*) as a more or less continuous "peripheral reticulum" (Kirby's fig. 220A,H) before disappearing--reminiscent of development in *N. hypertrophica* (List One, #3); this pattern seems the reverse of the *Nucleophaga* discussed under #2 (this list). Also, in the putative *Nucleophaga* now discussed (#5), what seemed to be an amoeboid body (early in development of the *Nucleophaga*) penetrated the host (*Trichonympha*) chromatin mass (i.e., nucleolus), possibly bearing some similarity to the plasmodial 'infection projection' illustrated by Karling (1977, p. 31, fig. 3)--see also mention of such in the third paragraph of our Discussion. Kirby (1941) noted that this apparent *Nucleophaga* (i.e., #5) is potentially distinctive. This organism should, if possible, be recollected and reinvestigated with present techniques. Fungal parasites of 'hypermastigote flagellates,' such as *Trichonympha*--and also 'devescovinid flagellates, such as *Caduceia* (#6, Lists One and Two)--should, as a matter of generally increasing knowledge of parasites of protozoa, be further inventoried.

6. *Nucleophaga* sp. (see Kirby, 1941). Kirby (1940) first mentioned, and later (Kirby, 1941) more fully discussed and illustrated (pp. 1055-1056), a *Nucleophaga* found in genus, *Caduceia*--a member of the Devescovinid Flagellates, a group of flagellates which are mainly commensalistic or mutualistic, 'xylophagous' organisms (Kudo, 1966) living in the intestines of kinds of termites, assisting (with the aid of bacteria) in digestion of microscopic bits of wood. Devescovinids are characterized (typically) by three anterior flagella, and one additional, often different appearing, trailing flagellum (cf. Hall, 1953); they thus are more broadly considered 'Trichomonads.' The nucleus is sometimes non-spherical (often cone-shaped or elliptical in *Caduceia*), anterior in the cell, and located at a specific position in relation to the flagellar apparatus, axostyle and parabasal body (see illustrations of various 'devescovinids' in Lee, 1985a). These things are pointed out here because--when infected by *Nucleophaga*, and due to structural limitations on uniform expansion of the nucleus--a peculiar hypertrophy (involving unilateral bulging, bending or contortion) of the *Caduceia* nucleus may occur (see Kirby's figs. 218K,L,N, p. 1055). Kirby's illustrations of spores (figs. 218 L,N) are interesting in that they appear to be on the large side for a *Nucleophaga*, but are perhaps more interesting because of his illustrations (L,M) of a distinct cytoplasm of each spore, the nucleus becoming eccentric in position. This seems to be a unique *Nucleophaga*, although *Nucleophaga* occurs in a range of Devescovinids (Kirby, 1941); parasites of Desvescovinids should, if possible, be evaluated in the light of molecular and electron-microscopic techniques.

7. *Nucleophaga* sp. In their publication, "The Biotic Association of Cockroaches," Roth and Willis (1960) mentioned--noting Mercier's publication (1907)--the occurrence of a fungus (*Nucleophaga*) in the nucleus of an amoeba, *Endamoeba blattae*, found in the lower digestive tract of certain cockroaches (e.g., *Blatta orientalis*, the oriental cockroach; and *Blatella germanica*, the German cockroach). Mercier (1907) was convinced of the identification of the nuclear parasite in *Amoeba* (= *Endamoeba*) *blattae* as *Nucleophaga* Dangeard, but seemed less certain that this 'species' (unnamed) was the same as Dangeard's *N. amoebae*. Dobell's (1919) review of the literature of potentially parasitic protozoa had also yielded mention of the occurrence of *Nucleophaga* in *Endamoba blattae* (citing Mercier's longer, 1910, publication). Kirby (1927) considered *Endamoeba blattae* distinct from *E. disparata* (#2, this list), though perhaps resembling it in early stages; *E. blattae* eventually demonstrates a distinctive, striated endoplasm (Kudo, 1966, fig. 190d)--more unusual than striated ectoplasm (for examples of the latter, see species of *Thecamoeba*; Bovee, 1985, pp. 172-173). Being found in different (distinct) amoeba hosts--and

(in this case) in different insect hosts--the *Nucleophaga* 'species' respectively in *E. blattae* and *E. disparata* are perhaps different, and should be reinvestigated. A *Nucleophaga* was also observed by Kirby (1927; his plate 26, fig. 61) in *Endamoeba simulans*. However, Kirby considered *E. simulans* similar to *E. blattae*--these two amoebas possibly housing similar *Nucleophaga* species.

LIST THREE: Organisms (or structures) considered, at one point or another, to possibly be *Nucleophaga*, but which we believe should be excluded from the genus.

1. Certain authors (e.g., Kirby, 1941; Dick, 2001) have appeared to conclude that Doflein (1907) observed an organism assignable to *Nucleophaga*. Karling (1977, p. 32) stated that Doflein (1907) "described and illustrated" motile cells (spores? gametes?) from "parasitized nuclei" of an amoeba (in this case, "*Amoeba vespertilio*")--these motile cells said to each possess an 'anterior,' short flagellum, and that such motile cells could fuse in pairs to form [both] biflagellate and amoeboid zygotes. The 'anterior' attachment of the flagellum of this 'organism' might seem reminiscent of the organism discussed by Kirby (1941; see #4, List Two). However, the small [short] forwardly curved flagellum described by Doflein (1907) on motile cells he observed does not match well with what is known of flagella of motile cells of either Chytridiomycota or Cryptomycota, which--though in some cases differing from each other in the point of flagellar attachment--are (in both groups) usually relatively long, and more or less trailing. Both Sparrow (1960) and Karling (1977) questioned if there was enough evidence to consider the organism described by Doflein (1907) a *Nucleophaga*. Dick (2001) listed Doflein's (1907) reference under *Nucleophaga peranemae* Hollande & Balsac, *seeming* to indicate he thought the organism Doflein observed (not named) would somehow prove (if named) to be a synonym of this species (but, *N. peranemae* spores are not known to be motile!). In any case, contrary to Karling's indication, we could not find that Doflein (1907) illustrated *the motile cells* he described. In examining Doflein's illustrations (his figs. 2 and 3, p. 17) of the microscopically 'granular appearing,' sometimes irregularly lobed, [non-flagellated] 'organism' he observed in *Amoeba vespertilio*--shown as occupying a significant portion of the trophic, amoeba cell--it seems unwise to assert that this is *Nucleophaga*; it does *not*, in fact, appear to be--although its true nature remains uncertain. It is possible that the 'organism' seen by Doflein (1907) was a rather large, supra-nucleolar, somewhat irregular, lobed, chromatin mass ('reticulum') of the amoeba; see discussion of a "chromatin reticulum" in *Amoeba vespertilio* (Calkins, 1933, p. 96). It is feasible, thus, that Doflein (1907) illustrated an internally 'diffuse,' pre-division stage of this amoeba--in which 'projections' of chromatin extend toward the periphery. In any case, the nature of the motile cells discussed by Doflein is unclear; these may have nothing directly to do with the amoeba in question, or anything parasitizing it.

2. Scherffel (1902), in perusing literature containing incidental findings of 'new' microscopic organisms, noted the occurrence (previously discussed and illustrated by Wildeman, 1898) of a possible *Nucleophaga* in the filamentous, conjugate alga, *Zygnema*. Scherrfel's (1902) account (of *Zygnema* allegedly containing *Nucleophaga*) was noted by Sparrow (1960). However, comments in both Scherffel (1902, p. 106) and Wildeman (1898, p. 118) suggest that another organism (potential parasite or scavenger?)--in addition to the supposed *Nucleophaga*--may have been present in the *Zygnema* 'host.' A telling point is that the nucleus of *Zygnema* was not seen at the same time as the parasite (or parasite complex)--unless it is the apparently deteriorated structure in Wildeman's fig. 3--indicating that the algal-cell nucleus had, in most cases, already been eliminated, and, accordingly, that this *Zygnema* 'host' was likely in a 'declining' state (substantiated by the often degenerated appearance of the *Zygnema* plastids; see Wildeman's figs. 1-5). Our further examination of Wildeman's plate 22 (figs. 1-7) suggests that [what appears to be] a protozoan (its apparent cysts capable of attaining larger size than a *Nucleophaga*) had invaded the, perhaps already 'damaged,' *Zygnema* cells; furthermore, the protozoan--possibly a *Pseudsopora*, a *Hyalodiscus*, or a *Vampyrellidium* (see, for example, Hall, 1953, pp. 222, 225)--was at some point invaded by a small, apparently fungal, parasite. The fact that this fungal parasite (sphaeroidal at first, e.g. Wildeman, figs. 2, 3, 5) can apparently attain an oval or elliptical shape (cf. Wildeman, figs. 1, 4), and may occur at various places in (even throughout) the protozoan cyst (e.g., Wildeman's figs. 1-3),

additionally indicates that the 'fungal organism' is not *Nucleophaga*, but more likely *Sphaerita* (see accounts of the morphology and occurrence of these genera in Sparrow, 1960; Karling, 1972, 1977). In any event, *Zygnema* does not seem to have been *directly* parasitized by the fungal organism in question. *Nucleophaga* is still not known, with certainty, to parasitize organisms other than types of protozoa.

3. Any truly cytoplasmic parasite, such as *Sphaerita* (and *Morella*, if recognized--cf. Karling, 1972--a non-flagellated entity otherwise resembling the allegedly flagellated *Sphaerita*), should not be considered equivalent to *Nucleophaga*. Various authors (e.g., Tyzzer, 1920; Karling, 1972, 1977) appeared to discuss such genera as *Nucleophaga* and *Sphaerita* as almost 'interchangeable' entities--assumed to differ mainly by 'position of occurrence' in the host-cell (nucleus vs. cytoplasm). Even as regards occurrence in 'kinds of' potential hosts, this presumption of 'essential similarity' (of *Nucleophaga* and *Sphaerita*) is encountered (e.g., Mattes, 1924; Sassuchin, 1934; Orenski, 1966; Sleigh, 1989). Sleigh, for example, did not clearly distinguish in precisely which organism(s) *Nucleophaga* occurred vs. that in which *Sphaerita* was found. However, the more that various organisms have been investigated, the more it has become apparent that the particular parasitic mode of 'nucleophagy' (vs. 'cytophagy') is a specific adaptation, involving utilization of the nucleolus in the case of *Nucleophaga* (Michel et al., 2009a, 2012; Corsaro et al., 2014, 2016). Some earlier authors (e.g., Lavier, 1935a) seemed to understand the obligate, unique relationship of *Nucleophaga* to the host nucleus. In any case, it is increasingly clear that what is *truly* a *Nucleophaga* (true nuclear parasite) cannot be equated to species of *Sphaerita* (or *Morella*).

DISCUSSION

As for species of *Nucleophaga*, we exclude (List Three) from the genus only those 'organisms' of which we feel certain of their exclusion. All named species (List One), in the current absence of convincing evidence to the contrary, are accepted for now as potentially distinct entities, as are additional forms (List Two, each there designated "*Nucleophaga* sp."). Contributing to our decision to recognize these 'taxa' (lists one and two) is the belief by various authors (e.g., Sassuchin, 1934; Kirby, 1941; Karling, 1977)--with which we agree--in a degree of host specificity of *Nucleophaga*; i.e., different hosts (different amoebae, and certain other protozoa) may house different species of this nuclear parasite. There are probably a number of [perhaps many] species of *Nucleophaga*, although they may indeed resemble one another (cf. Hollande and Balsac, 1941). Such a view would seem supported by the determination (Corsaro et al., 2016) that *Nucleophaga terricolae* and *Nucleophaga amoebae* (cf. Corsaro et al., 2014)--though quite similar in appearance (especially under the light microscope)--are nonetheless molecularly distinct (5.5 % genetic difference). Following this logic, we are, for example, considering *Nucleophaga intestinalis* (Brug, 1926) and *N. hypertrophica* (Epstein, 1922) as potentially distinct, even though various authors (e.g., Brumpt and Lavier, 1935; Dick, 2001) considered them the same (and that *N. intestinalis* was synonym of *N. hypertrophica*). Part of why we do not, presently, accept this synonymy is that Epstein (1922) and Brug (1926) did their original studies of *Nucleophaga* in different parasitic human amoeba--*Endolimax nana* (cf. Epstein), and *Iodamoeba buetschlii* (cf. Brug). We feel it is not possible to be certain that these (and other species we are putatively recognizing) are identical; also, there may be a difference in the effect of these two parasites on the nucleolus of the host (see #3, #4, List One).

Two types of development have been *alleged* for *Nucleophaga*--discussed by Lavier (1935b), and summarized by Kirby (1941). In the *first* type, the enlarging thallus (multinucleate 'plasmodium' or 'sporangium') of *Nucelophaga* is bordered externally by a (sporangial) membrane, and there is a more or less synchronous development of spores within the 'sporangium.' This is the type of development observed by Dangeard (1895) in *Nucleophaga amoebae* (found in the free-living '*Amoeba verrucosa*,' = *A. proteus* according to Pénard, 1905; cf. Kirby, 1941). This is also the mode of development illustrated by Kirby (1941, his fig. 218B-E, p. 1055) for *Nucleophaga* sp. found in *Endamoeba disparata* (occurring in kinds of termites; Kirby, 1927), and appears to be that illustrated by Hollande and Balsac (1941, p. 40, fig. 6) for *Nucleophaga peranemae*. In the *second* type of development, there is *seemingly* no (membrane-

bounded) multinucleate structure (i.e., no 'plasmodium' or 'sporangium' *per se*), and the spores are apparently the product of non-synchronous divisions. This 'second type' is said to be that observed by Epstein (1922) and by Brug (1926) in two different human amoebal parasites, and by Sassuchin (1934) in an amoeba (*Entamoeba citelli*) found in ground-squirrels (see Kirby, 1941, p. 1054). Kirby (1941), though, questioned that there could be two such distinct developmental patterns in *Nucleophaga*--raising the possibility that this apparent difference might be a consequence of misinterpretations, or differing interpretations, of what was seen [or, we might add, whether observations were made from stained slides or living material]; also, the exact stage of the *Nucleophaga*, when observed, could play a role in precisely what was concluded. It is plausible there is merely a spectrum of variation, based on one developmental theme. As regards variation, Lavier (1935b) noted that development in *Nucleophaga ranarum* (in amoebae of amphibians) was similar to the first type of development, *except* that formation of spores was non-synchronous (as in the second type of development)--in other words, an example exhibiting aspects of both supposed types of development. In our opinion, an organism [*Nucleophaga*, or any other], regardless of differences in life cycle, would necessarily possess a plasma membrane.

As regards (the activity of) amoeba hosts, freed spores of *Nucleophaga* are apparently engulfed by phagocytosis (forming a 'food vacuole' around them)--much as any potential food particle (Sassuchin, 1934; Karling, 1977; Corsaro et al., 2014, 2018). These 'engulfed spores,' however, are not digested, subsequently 'migrating' (more likely carried passively, as by protoplasmic streaming) to the host nucleus, penetrating the nucleus and perhaps also the nucleolus (Karling, 1977, attributed this observation to Dangeard, 1895). Karling (1977, fig. 3, p. 31) illustrated an apparently labile 'infection projection' [as we term it] from the spore's cytoplasm. There is also some suggestion, however, that an 'early plasmodial stage' may penetrate the nucleus--see Kirby's (1941) discussion of Greeff (1866); see also Lavier (1935b, p. 357) concerning what is possibly an early plasmodial invasion stage. In any event, the precise sequence of events, once *Nucleophaga* is within the nucleus of the host, has been the subject of discussion--probably involving some variation (differences). Brug (1926) stated that, in the case of *N. intestinalis*, *Nucleophaga* developed *beside* the nucleolus, subsequently flattening it (or its remains) against the nuclear membrane; this also seems to be the case in the *Nucelophaga* sp. found in *Pseudospora* (see Karling's, 1977, fig. 22, p.31), as well as that observed by Tyzzer (1920, his fig. 14) in *Pygolimax*. Concerning an amoeba in termites, Kirby (1927) observed that the *Nucleophaga* parasite, when within the nucleus of the amoeba, first surrounds the nucleolus, 'centralizing' it, before its eventual destruction; however, the reverse of this process, i.e., a 'peripheralization' of chromatin (nucleolar material), seems to occur in the case of the *Nucleophaga* found in *Trichonympha* (Kirby, 1941), and perhaps also in *Nucleophaga hypertrophica* (Epstein, 1922, see his fig. 6). Regardless of the precise mechanism utilized by various *Nucleophaga* species, it seems that chromatin material [of the nucleolus of the host] is eventually depleted or eliminated (Kirby, 1941). Some insight into the details of nucleolar depletion is found in Corsaro et al. (2014, 2016) in which it was confirmed the *Nucleophaga* parasite grows at the expense of the host karyosome (= endosome = nucleolus); in fact, minute finger-like extensions were observed on a 'trophic stage' of *Nucleophaga* (preceding the 'endospore stage), indicative of the ability of *Nucleophaga* (albeit perhaps in a 'residual' form) to perform phagocytosis; remnants of host nucleolar material were found inside the *Nucleophaga* cytoplasm (Corsaro et al., 2014; see their figs. 1d,h).

Nucleophaga was, from the time of its description (Dangeard, 1895), considered a chytrid-like organism. In spite of being considered 'fungal,' *Nucleophaga* was omitted by Fitzpatrick (1930), Clements and Shear (1931) and Bessey (1950)--odd omissions, since these authors included *Sphaerita* (a genus thought similar to *Nucleophaga*, but which infects cytoplasm of the host rather than the nucleus; cf. Sparrow, 1960). When *Nucleophaga was* included in coverage, it was typically placed in family Olpidiaceae (Chytridiales)--see Sparrow, 1960 and Karling, 1977--near *Sphaerita*. Both Sparrow and Karling (see also Karling, 1972), at some point, questioned the generic relationships of *Nucleophaga*. Dick (2001) merely included *Nucleophaga* among a mélange of "miscellaneous genera"--some very possibly unrelated. Corsaro et al. (2014), based on molecular and microscopic evidence, considered

Nucleophaga to represent a distinct lineage within the Cryptomycota (viz. "Rozellomycota" of Corsaro et al., 2014)--having morphologically, though in diminished form, a *Rozella*-like trophic stage (see p. 4494 in Corsaro et al., 2014). This viewpoint was modified in Corsaro et al. (2016) in which the "Rozellomycota" and the Microsporidia were suggested to be evolutionarily connected; *Nucleophaga* seemed to fit into this revised picture as a somewhat intermediate [morphologically and genetically] entity, which, based on updated molecular findings, formed a sister clade (rather obscurely named "Nucleophagales," cf. Corsaro et al., 2018, their "Introduction") with Microsporidia--being perhaps more distantly related to *Rozella*. Possible further clarification came when Bass et al. (2018) determined *Nucleophaga* to be a distinct lineage within an expanded (diverse) Microsporidia; not just molecular evidence, but TEM studies (demonstrating such microsporidian features as polar filaments, an anchoring disc, loss of flagellation, and lack of mitochondria) appeared to confirm this (Corsaro et al., 2016; Bass et al. 2018); *Rozella* (by contrast) has mitochondria--although depauperate in the thallus (James et al., 2013), compared with the more robust, single mitochondrion in the *Rozella* zoospore (Held, 1975). Bass et al. (2018) viewed the Rozellida as a more restricted group (centering mainly on *Rozella*) than, for example, did Corsaro et al., 2014, 2016). Some authors (e.g., Corsaro et al., 2018), however, continued to view the "Rozellomycota" more broadly--considering it to encompass Microsporidia, *Nucleophaga*, and *Rozella* (*Rozella* connected basally to both the preceding)--suggesting as well that Rozellids constitute an early chytrid lineage, and that Microsporidia also evolved from Rozellids (see James et al., 2013, re: the potential connection of Microsporidia and Cryptomycota). In any case, *Nucleophaga* has closer relationships to Microsporidia and Cryptomycota than to Chytridiomycota (i.e., as presently recognized). It has recently been suggested (Blackwell et al., 2016, 2017) that certain other genera, e.g., *Plasmophagus* and *Dictyomorpha*--once considered to be in family Olpidiaceae (Chytridiales), along with *Nucleophaga* and *Sphaerita*--may as well have closer relationships to Cryptomycota than to Chytridiomycota.

Corsaro et al. (2018) pointed to the need for continuing study of amoebophagous organisms (such as *Nucleophaga*), as these are likely to prove key to understanding early evolution of fungal groups, and primitive animals (i.e., kinds of 'protozoa'). Not only molecular studies (e.g., Corsaro et al., 2014, 2016; Quandt et al., 2017; Bass et al., 2018), but those of electron microscopy (e.g., Michel et al., 2009a, 2009b, 2012) are important to ascertain the precise nature (even proper identification) of the organisms, the mode of action (exact nature of the parasitism), and the life cycles of such primitive forms (how they are able to survive and function in their hosts)--explaining such facts as, for example, that *Nucleophaga* has morphological/functional characteristics of both *Rozella* (e.g., phagocytic ability, involving small pseudopodia; Powell et al., 2017) *and* of Microsporidia (e.g., polar filaments and adhesions discs; discussed above). It is important to understand that *Nucleophaga*--though quite small (0.4 to 2.5 µm), and typically with no more than slight mobility of its own, at any point--does indeed have a 'trophic stage' (elucidated by electron microscopy; see Corsaro et al., 2014, 2016) preceding the more familiar 'endospore stage;' this had proved essentially impossible to determine with light microscopy, because of the tiny and seemingly uniform nature of the coccoid bodies of the parasite. *Nucleophaga* may now be understood as a kind of morphological 'missing link,' as well as representing 'molecular intermediacy,' among groups within which its relationships are now understood to lie (see preceding paragraph). Needed are more studies (such as those of Corsaro et al. and Michel et al., mentioned above) of additional taxa of *Nucleophaga*--and, of possibly related organisms such as *Sphaerita* (and even *Morella*, which has never been nomenclaturally validated, cf. Karling, 1972, 1977; and which may also be a later homonym, cf. Dick, 2001). Such desired studies invoke the necessity of, first, collecting and screening of protozoan hosts potentially containing new or poorly known parasitic (or 'hyperparasitic') organisms.

It would be well to re-collect any entities discussed in our Lists (One, Two) that potentially represent taxa additional to the two species (List One) already well-studied by Corsaro et al. (2014, 2016; molecular analysis). Further possibilities for study, perhaps less well known, are sporadically found in the literature--e.g., Ghosh (1970) who noted the find (Wenrich, 1940) of a possible *Nucleophaga* in *Entamoeba muris* (occurring in the caecum of mice and rats), and Garcia (2007) who appeared to indicate

occurrence of *Nucleophaga* sp. in *Entamoeba coli* (in humans). It can admittedly sometimes be difficult, especially in older literature, to distinguish the presence of *Nucleophaga* from what may be merely a division preparatory stage of the host organism, e.g., in which so-called 'chromidia' are found, or even odd, stained, ingested particles (see examples, figs. 89E and 90B of *Entamoeba coli* and *E. histolytica* respectively, in Minchin, 1922). Regardless, any newly found (or 'found again') taxa of *Nucleophaga* should be reported, even if left unnamed. Also, better understanding of the life cycle of *Nucleophaga* may accrue from such investigations. That nucleophagy (especially involving utilization of the nucleolus) is a specific adaptation--pointing to distinction of *Nucleophaga* as a genus--has been discussed (List Three, #3). However, as mentioned, the details of nucleolar utilization may vary between (perhaps even within) species. The rare occurrence of *Nucelophaga* in the cytoplasm (cf. Kirby, 1941, pp. 1053-1054; Karling, 1972, p. 227)--in a more 'separated' arrangement than the typically compact ('morula') configuration--probably means, due to parasitism (and hypertrophy) of the nucleus by *Nucleophaga*, that the nuclear membrane has broken down (become "invisible," Sassuchin, 1934, p. 218); the *Nucleophaga* spores, 'released' from the nucleus, are thus consequently in (perhaps appearing to have originated in) the cytoplasm (cf. Tyzzer, 1920, p. 204; Brug, 1926, p. 467; Kirby, 1927, p.197). In other words, it is during this stage that *Nucleophaga* spores are in the initial 'process' of being 'dispersed,' albeit passively--subsequently entirely from a [dead or dying] host undergoing [eventual] disintegration (Sparrow, 1960, p. 123). Further observations of life cycles of *Nucleophaga* and *Sphaerita* (and related organisms) are needed to determine, with certainty, aspects of development and successive events (e.g., dispersal).

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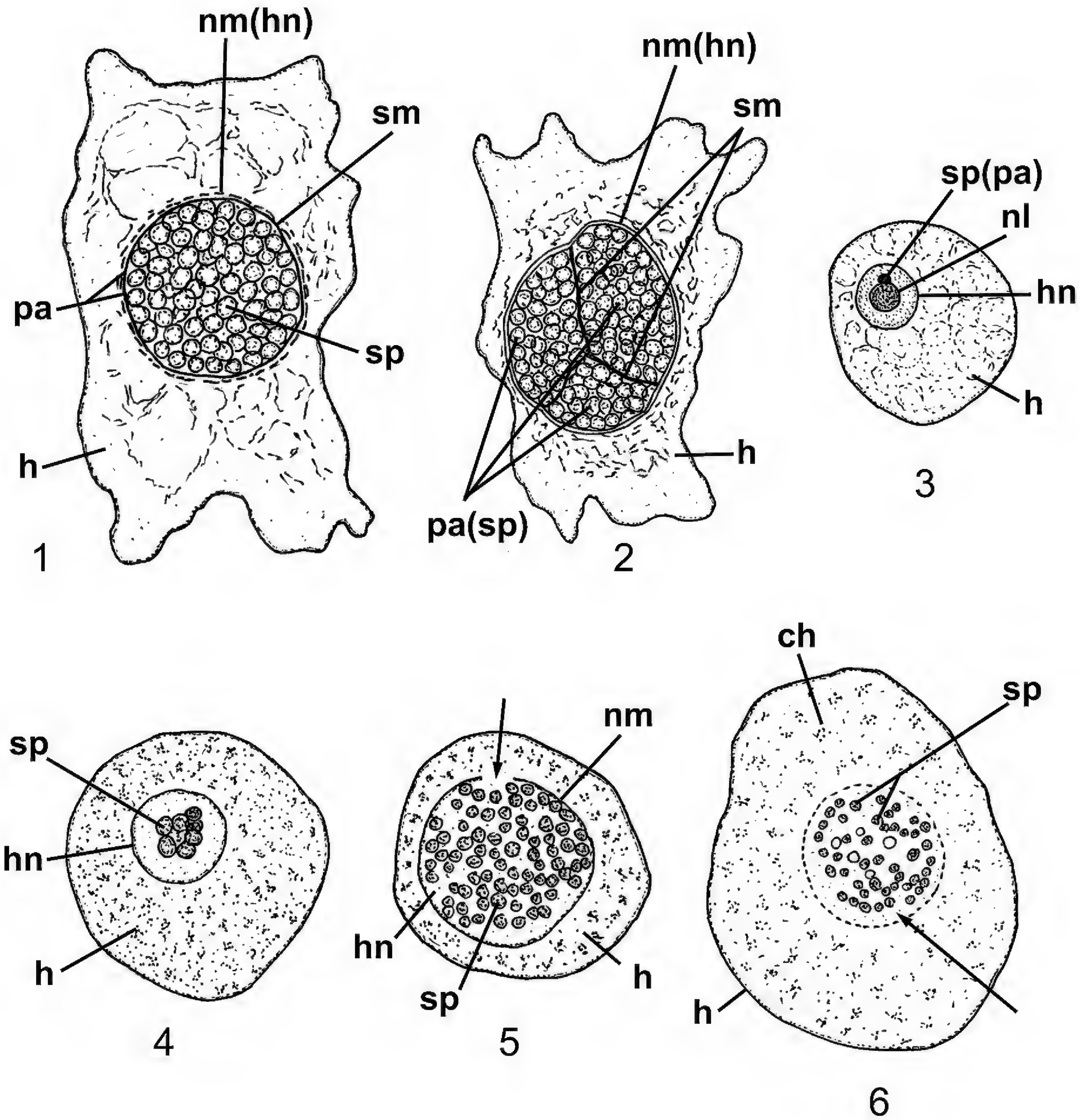
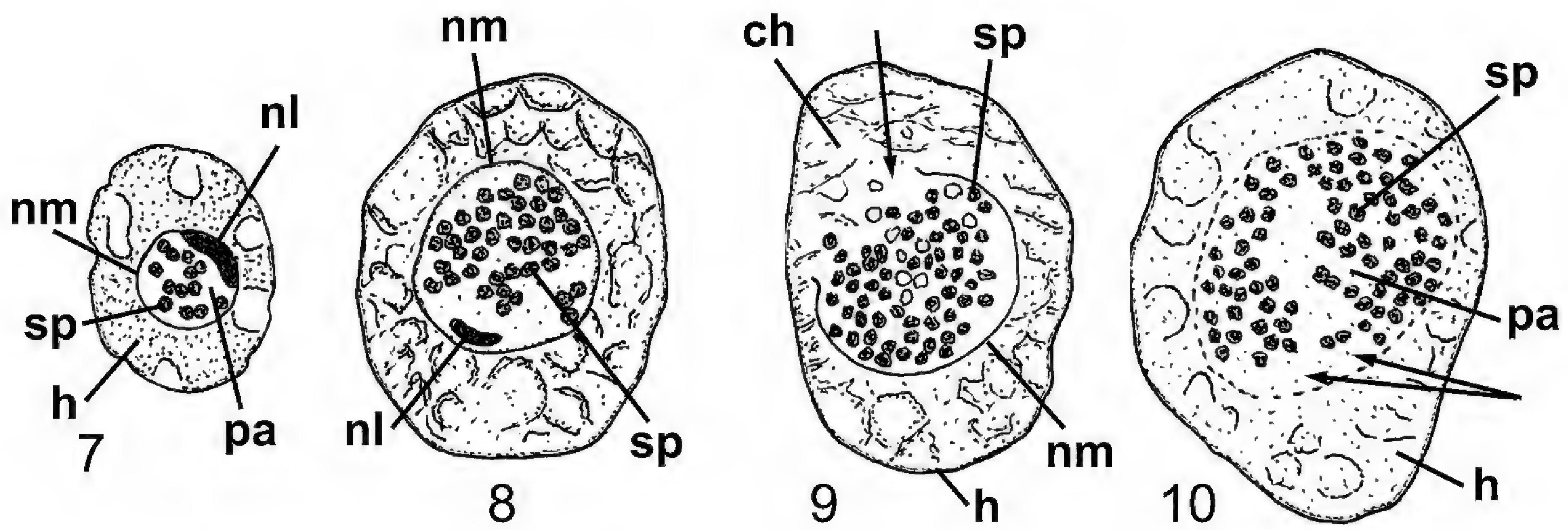
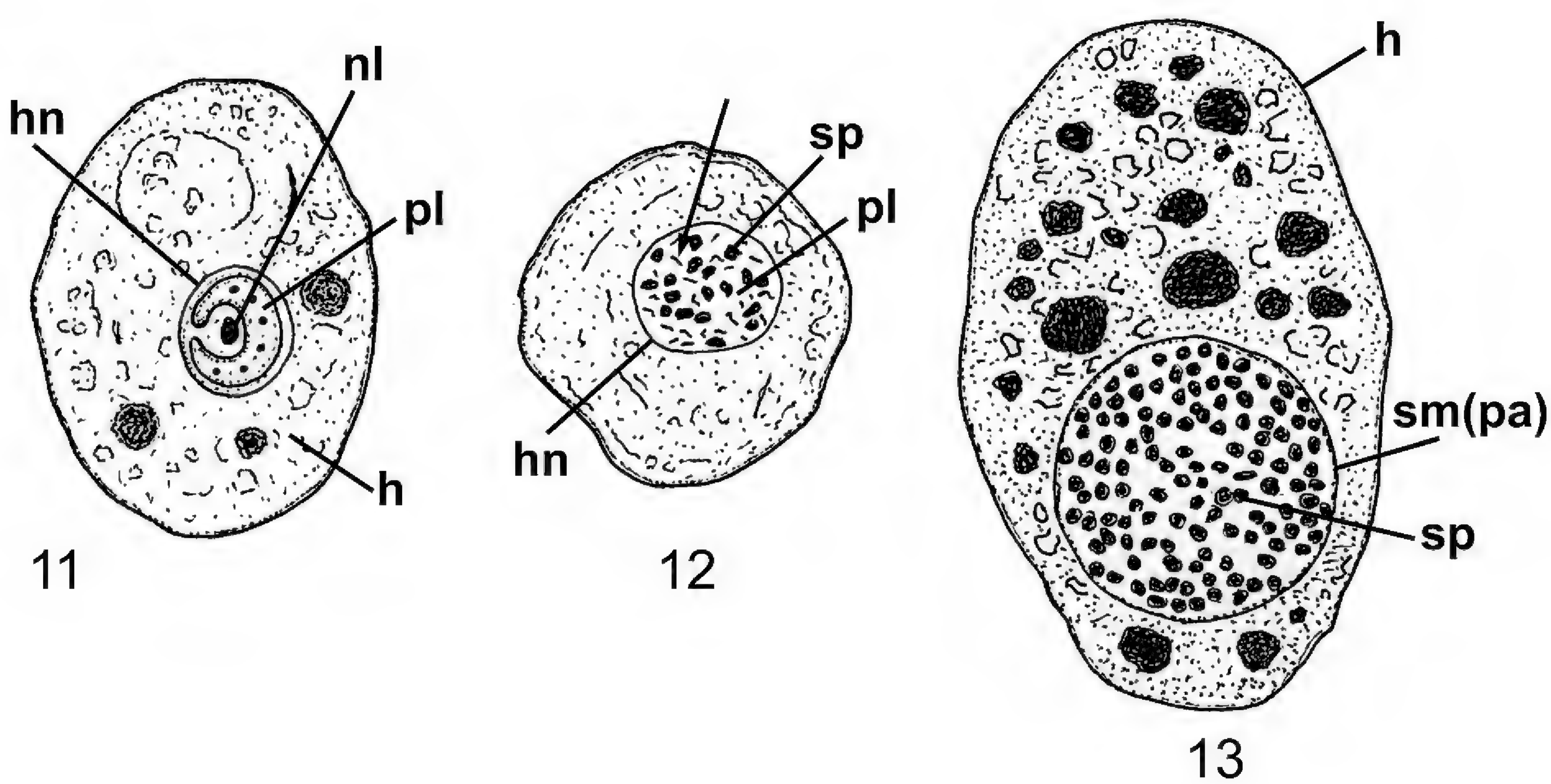
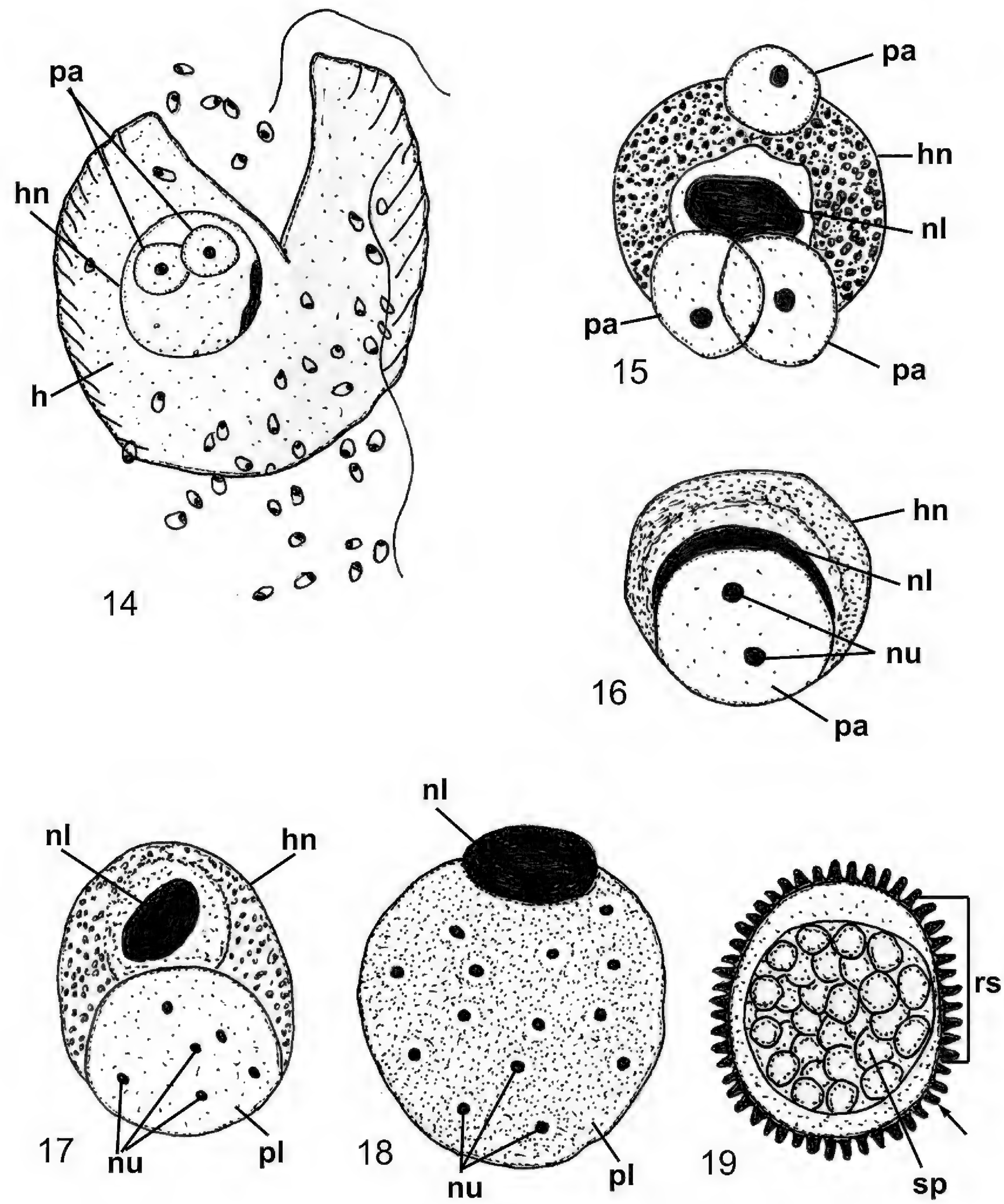


Figures 1-2. Parasite (pa), *Nucleophaga amoebae* (after Dangeard, 1895); host (h), *Thecamoeba verrucosa*. **Fig. 1**: Coccoid bodies are spores (sp), i.e., endospores of the parasite; note sporangial membrane (sm) of the parasite. Host nucleus (hn) represented by remnants of its nuclear membrane (nm). **Fig. 2**: Three parasites, tightly packed--each with its mass of spores (sp) and sporangial membrane (sm)--occupying hypertrophied host-nucleus (hn) of which only its membrane (nm) remains. **Figures 3-6**. *Nucleophaga hypertrophica* (pa), after Epstein (1922); host (h), *Endolimax nana*. **Fig. 3**: Spore (sp) infecting the nucleolus (nl), inside nucleus (hn) of host. **Fig. 4**: Small group of spores (sp) has arisen, centrally, obliterating the nucleolus. Nucleus (hn) of host beginning to undergo hypertrophy. **Fig. 5**: Mass of spores (sp) now occupies the significantly hypertrophied host nucleus (hn); note ruptured (arrow) nuclear membrane (nm) of host; relative area of host reduced. **Fig. 6**: Group of (endo-) spores (sp); nucleus (including its membrane) of host obliterated; some 'clear space' (arrow) evident around mass of spores; no sporangial membrane present; host cytoplasm (ch) seemingly intact, but shows hypertrophy.





Figures 7-10. Parasite (pa), *Nucleophaga intestinalis* (after Brug, 1926); host (h), *Iododamoeba buetschlii*. **Fig. 7**: Relatively young stage of parasite (but no sporangial membrane evident); spores (sp), infecting host nucleus (nuclear membrane, nm, present--nucleolus, nl, flattened against it). **Fig. 8**: Somewhat older; number of spores (sp) increased. Host nucleus hypertrophied, represented by the nuclear membrane (nm) and flattened nucleolus (nl). **Fig. 9**: Nucleolus no longer evident. Note rupture (arrow) of nuclear membrane (nm) of host and passive release of endospores (sp) into cytoplasm (ch) of host. **Fig. 10**: Older; host nucleus destroyed. Note clear space (arrows) potentially 'separating' parasite and host (sporangial membrane lacking around spores, sp). **Figures 11-13**. *Nucleophaga ranarum* (pa), after Lavier, 1935b; host (h), *Entamoeba ranarum*. **Fig. 11**: Young plasmodial-like structure (pl)--an apparent early infection stage--surrounding remains of nucleolus (nl), inside nucleus (hn) of host. **Fig. 12**: nuclei of 'plasmodium' (pl) converted to spores (sp); pieces (arrow) of interior of host nucleus (hn) scattered. **Fig. 13**: Older; nuclear area hypertrophied; nucleus destroyed. Sporangial membrane (sm) of parasite still evident. Spores (sp) 'multiplied' in presumably non-synchronous fashion. Host generally hypertrophied.



Figures 14-19. Parasite (pa), *Nucleophaga peranemae* (after Hollande and Balsac, 1941); host (h), *Peranema trichophorum*. **Fig. 14**: Two smallish parasites developing in host-nucleus (hn). **Fig. 15**: Illustrates only the nucleus of the host (hn), nucleolus (nl) inside, three young parasites developing. **Fig. 16**: Host nucleus (hn), with single parasite inside--i.e., an incipient plasmodium which divided (internally) forming two nuclei (nu). 'Dark cap' is host nucleolus (nl) flattened, pushed toward one side. **Fig. 17**: Developing plasmodium (pl), several nuclei (nu) now evident. As may be judged, the exact stage at which the nucleolus (nl), of the host-nucleus (hn), is flattened or pushed aside, varies. **Fig. 18**: Only the parasitic plasmodium (pl) shown; more nuclei (nu) now evident, each potentially becoming a spore; nucleolus (nl) of host-nucleus in process of being flattened, but persisting for a time. **Fig. 19**: Resting spore (rs) or 'cyst;' note spore-wall with short spines (arrow), and note mass of spores (sp) internally.

The effects of plant growth regulators on the growth and yields of hydrocarbons in *Helianthus annuus* cv. Munchkin (Asteraceae, Sunflowers)

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ABSTRACT

Sunflowers, *H. annuus* cv. Munchkin, were subjected to 11 different treatments to determine their effects on the free, stored hydrocarbons (HC) concentrations, leaf biomass, and yields of HC/ g biomass. Biomass was significantly larger than the control for plants subjected to mechanical leaf injury or spraying with benzothiadiazole (1000 ppm). In contrast, spraying with methyl jasmonate (100 µM), gibberellic acid (100 µM), or indole-3-acetic acid (100 µM), resulted in significantly less biomass. Mechanical leaf injury or spraying with Chlormequat Cl (1000 ppm) gave % HC yields the same level as the control. But, % HC yields were significantly lower than the control for plants sprayed with methyl jasmonate (100 µM), gibberellic acid (100 µM), indole-3-acetic acid (100 µM), or Ethephon (100 ppm) (produces ethylene). Total HC yields (as g HC/ g dry wt. 10 lvs.) was correlated with biomass and % HC yields for which mechanical leaf injury or spraying with Chlormequat Cl (1000 ppm) or 2,4-D (100 ppm) resulted in g HC statistically equal to the control plants. However, spraying with methyl jasmonate (100 µM), gibberellic acid (100 µM), indole-3-acetic acid (100 µM), or Ethephon (100 ppm) gave g HC yields significantly lower that the control. Over all, none of the treatments enhanced HC production significantly larger than the control. This may be due to the mixture of chemical classes such as terpenoids, lipids, waxes and sterols that are controlled by genes in various conflicting pathways.
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KEY WORDS: *Helianthus annuus,* Sunflower, methyl jasmonate, effects on hydrocarbon yields.

In a seminal paper on the induction of sesquiterpene lactone (STL) defenses in *Helianthus annuus*, by surface application of methyl jasmonate (MeJA), Rowe, Ro and Rieseberg (2012) found that MeJA treated sunflower plants had a lower STL production and lower glandular trichome density. This is in contrast to other studies that have found MeJA to induce increased concentrations of terpenoids in cotton (*Gossypium hirsutum*, Opitz, Kunert and Gershenzon, 2008), *Tanacetum parthenium* (Majdi et al. 2015) and see review on the roles of MeJA in plants by Browse (2005).

It appears that defense chemicals are both constitutive and inducible defenses (see Wittstock and Gershenzon, 2002 for discussion). Recently, we reported (Adams et al. 2017c) that progeny of high hydrocarbon (HC) yielding sunflower (*H. annuus*) populations displayed much reduced HC yields when grown in greenhouse conditions. Notice (Fig. 1) that the percent HC (greenhouse / field grown HC yields) decreased to 45.9, 55.6 and 78.3%. In addition, g HC / g DW leaves was very reduced to from 6.1 to 17.9% in greenhouse grown plants. It appears that biotic and abiotic factors in natural populations can have large effects on HC yields. With this in mind, it seemed of interest to investigate various plant growth regulators on HC yields from greenhouse grown Munchkin, a dwarf sunflower cultivar.

This is a part of a continuing study on the development of sunflowers as a source for natural rubber and bio-fuels from the biomass (Adams et al., 1986; Adams and Seiler, 1984; Adams and TeBeest, 2016; Adams et al. 2016; Adams and TeBeest, 2017; Adams et al. 2017a,b,c; Adams et al. 2018a,b,c; Pearson et al., 2010a,b; Seiler, Carr and Bagby, 1991,).

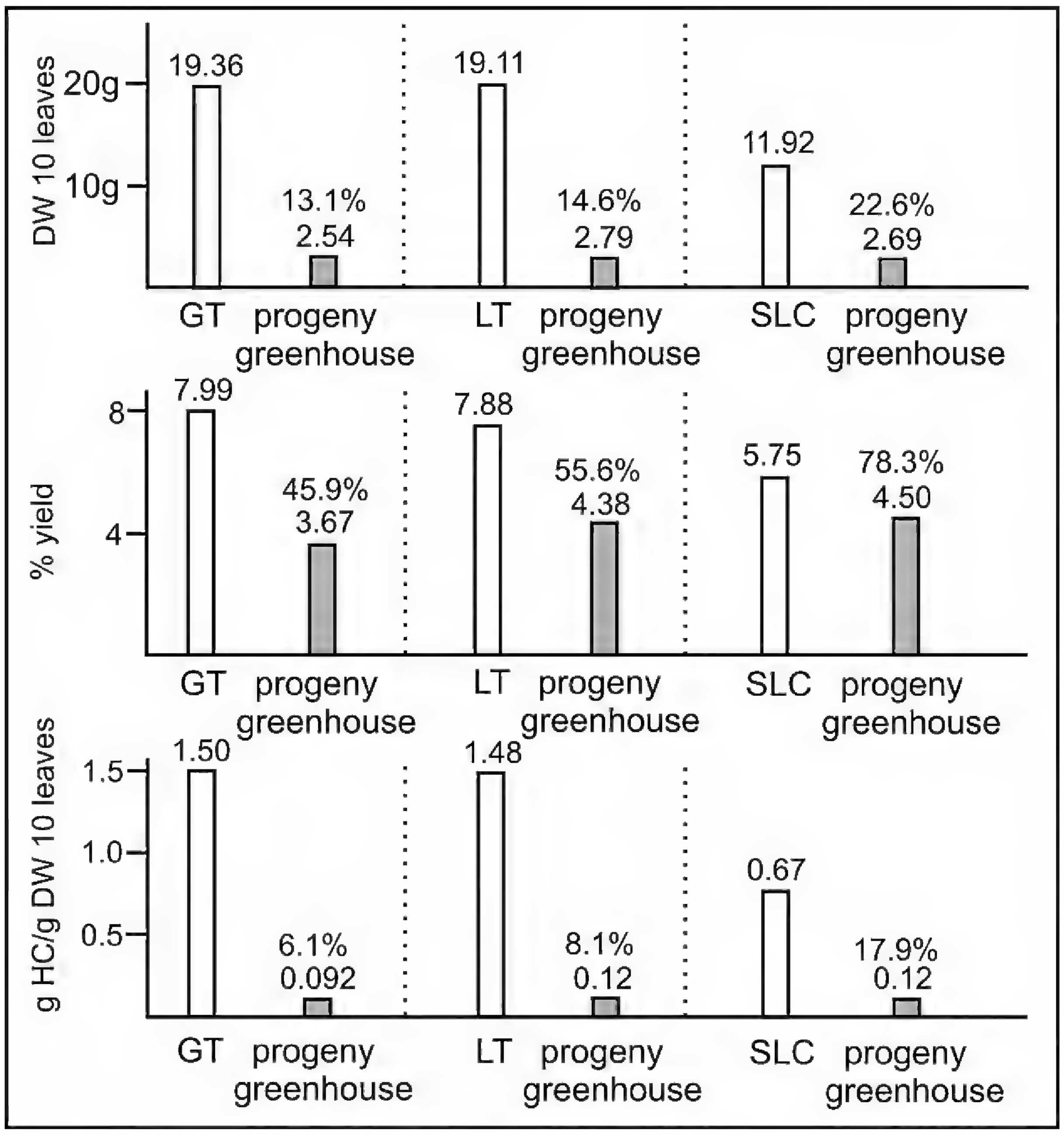

Figure 1. Comparison of DW 10 leaves, HC yield and g HC/ gDW 10 leaves for field sampled sunflowers from Gruver, TX (GT) vs. their progeny grown in the greenhouse at OPSU under ambient natural light (adapted from Adams et al. 2017c).

MATERIALS AND METHODS

Seeds of *H. annuus* cv. Munchkin were obtained from Sunflower Selections, Inc., Woodland, CA. Seeds were planted in 6 " square plastic pots using Miracle Grow® potting soil. Plants were grown in a growth chamber with LED lighting approximately equal to daylight for 16 hr light, 8 hr dark cycles and watered as needed.

The 10 lowest growing, non-yellowed, mature leaves were collected at stage R1 when the first flower but appeared. The leaves were air dried in paper bags at 49° C in a plant dryer for 24 hr or until 7% moisture was attained.

Leaves were ground in a coffee mill (1mm). 3 g of air-dried material (7% moisture) were placed in a 125 ml, screw cap jar with 20 ml hexane, the jar was sealed, then placed on an orbital shaker for 18 hr. The hexane soluble extract was filtered through a Whatman paper filter into a pre-weighed aluminum pan and the hexane evaporated on a hot plate (50°C) in a hood. The pre-weighed aluminum pan with concentrated hydrocarbon extract was weighed and tared. Extraction of identical samples by shaking and soxhlet (8 hr) yielded a correction factor of 1.9 (soxhlet yield/ shaking yield), which when corrected to oven dry weight basis (ODW) by 1.085 resulted in a total correction factor of 2.06.

ANOVA and SNK (Student Newman-Keuls) multiple range tests were programmed following the formulations in Steel and Torrie (1960).

TREATMENTS WITH GROWTH REGULATORS

At the first bud stage, Munchkin plants were treated as shown in Table 1.

Table 1. Treatment of sunflower (cv. Munchkin) with growth regulators and mechanical injury.

Treatment	ref.	Code	Reported effects on plants	# plants
Control		CT		7
Methyl jasmonate (100uM), sprayed	1,2,3	MJ	Increased parthenolide in *Tanacetum parthenium;* xanthumin in *Xanthium*.	7
Mechanical injury w/ cloth wheel, 4 lines	4	IN	Induction of terpenoids in plants.	7
γ-aminobutyric acid GABA,100 ppm	5	AB	Increased defensive enzymes in *Helianthus*.	7
Paclobutrazol 150 ppm	6,7	PB	Growth retardant. Increased pyrethrins in *Chrysanthemum*.	7
BZTD 1000 ppm benzothiadiazole	8	BZ	Induced synthesis of scopoletin in sunflower.	7
2,4-D, 2,4-dichlorophenoxyacetic acid, 100 ppm	9	24D	Accumulation of scopolin in sunflowers. Common tissue culture hormone.	7
Ethephon, (= Floral), 100ppm	6	ET	Decrease apical growth, promote branching. Flower abortion. Delayed flowering	7
Indole-3-acetic acid (IAA), 100 μM	3,8	IA	Increased phenolics in Brassica. Increased xanthumin in *Xanthium*.	7
Gibberellic acid, GA3, 100 μM	3	GA	Increased xanthumin in *Xanthium*.	7
Salicylic acid, 1000 ppm	1,5,8	SA	Increased oil content in *Brassica*. Increased defensive enzymes in *Helianthus*. Increased parthenolide in *Tanacetum parthenium*.	7
Chlormequat chloride, 1000ppm	6,7	CC	Suppresses stem elongation. Increased pyrethrins in *Chrysanthemum*.	7

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Treatment with growth regulators has had a long history of producing morphological and chemical changes in plants as is shown in the papers referenced in Table 1. Because this study was a screening project, the quantities utilized were those frequently utilized from the literature and not optimized.

RESULTS

Table 2 shows the results from the treatments ANOVA and SNK statistical analyses. Differences for biomass (g dw 10 lvs/ plant) among treatments were very highly significant ($P = 0.37^{-3}$ **). Biomass was significantly larger than the control for mechanical leaf injury or spraying with benzothiadiazole (1000 ppm). In contrast, spraying with methyl jasmonate (100 µM), gibberellic acid (100 µM), or indole-3-acetic acid (100 µM), resulted in significantly less biomass. Mechanical leaf injury or spraying with Chlormequat Cl (1000 ppm) gave % HC yields the same level as the control. But, % HC yields were significantly lower than the control for plants sprayed with methyl jasmonate (100 µM), gibberellic acid (100 µM), indole-3-acetic acid (100 µM), or Ethephon (100 ppm) (produces ethylene). Total HC yields (as g HC/ g dry wt. 10 lvs.) was correlated with biomass and % HC yields for which mechanical leaf injury or spraying with Chlormequat Cl (1000 ppm) or 2,4-D (100 ppm) resulted in g HC statistically equal to the control plants. However, spraying with methyl jasmonate (100 µM), gibberellic acid (100 µM), indole-3-acetic acid (100 µM), or Ethephon (100 ppm) gave g HC yields significantly lower that the control. Over all, none of the treatments enhanced HC production significantly larger than the control. This may be due to the mixture of chemical classes such as terpenoids, lipids, waxes and sterols that are controlled by genes in various conflicting pathways.

Table 2. Comparison of dry weight (10 leaves), percent HC yields, and g HC/ gDW 10 leaves for cv. Munchkin, subjected to 12 treatments and analyzed after 4 days. Mean valuess with the same suffix letter are not significantly different (P= 0.05). Treatment codes: see Table 1.

biomass	IN	BZ	24D	AB	CT	ET	PB	SA	CC	GA	IA	MJ	F ratio significance
wt. 10 leaves	3.37 a	3.33 a	3.25 ab	3.21 ab	3.18 ab	3.18 ab	3.14 ab	3.06 ab	3.02 ab	2.90 abc	2.70 bc	2.53 c	F= 3.86 $P = 0.37^{-3}$ ***
treatment/ HC yield	CT	CC	IN	24D	BZ	SA	PB	AB	MJ	IA	GA	ET	F ratio significance
% HC yield	4.66 s	4.48 s	4.45 s	4.28 st	4.12 st	3.96 st	3.88 st	3.70 st	3.26 tu	3.21 tu	3.10 tu	2.49 u	F= 5.76 $P = 0.15^{-4}$***
treatment gHC yield	CT	IN	24D	CC	BZ	SA	AB	PB	GA	IA	MJ	ET	F ratio significance
g HC/ g 10 leaves	.149 x	.149 x	.139 x	.136 x	.134 x	.121 xy	.120 xy	.119 xy	.090 yz	.087 yz	.081 z	.080 z	F= 8.08 $P = 0.117^{-5}$***

Graphing the yields by treatment reveals some interesting patterns (Fig. 2). The control (CT) is highest in % HC yield, gHC/ g10 lvs (biomass), and, statistically, in the highest group for biomass. Mechanical injury (IN) was near the maximum for all variables (Fig. 3). Chlormequat chloride (CC) was near the maximum in both %HC yields and gHC/ g 10 lvs. (Fig. 2).

Several treatments (Ethephon, ET; Gibberellic acid, GA; Indole-3-acetic acid, IA; Methyl jasmonate, MJ) produced significantly lower amounts of %HC yields and gHC/ g10 lvs. (Fig. 2).

Ethephon (ET) and γ-aminobutyric acid (AB) both reduced the %HC yields and gHC/ g 10 lvs, but had little effect on biomass (Fig. 2). Indole-3-aceetic acid (IA) and methyl jasmonate (MJ) both reduce the biomass in only 4 days after treatment.

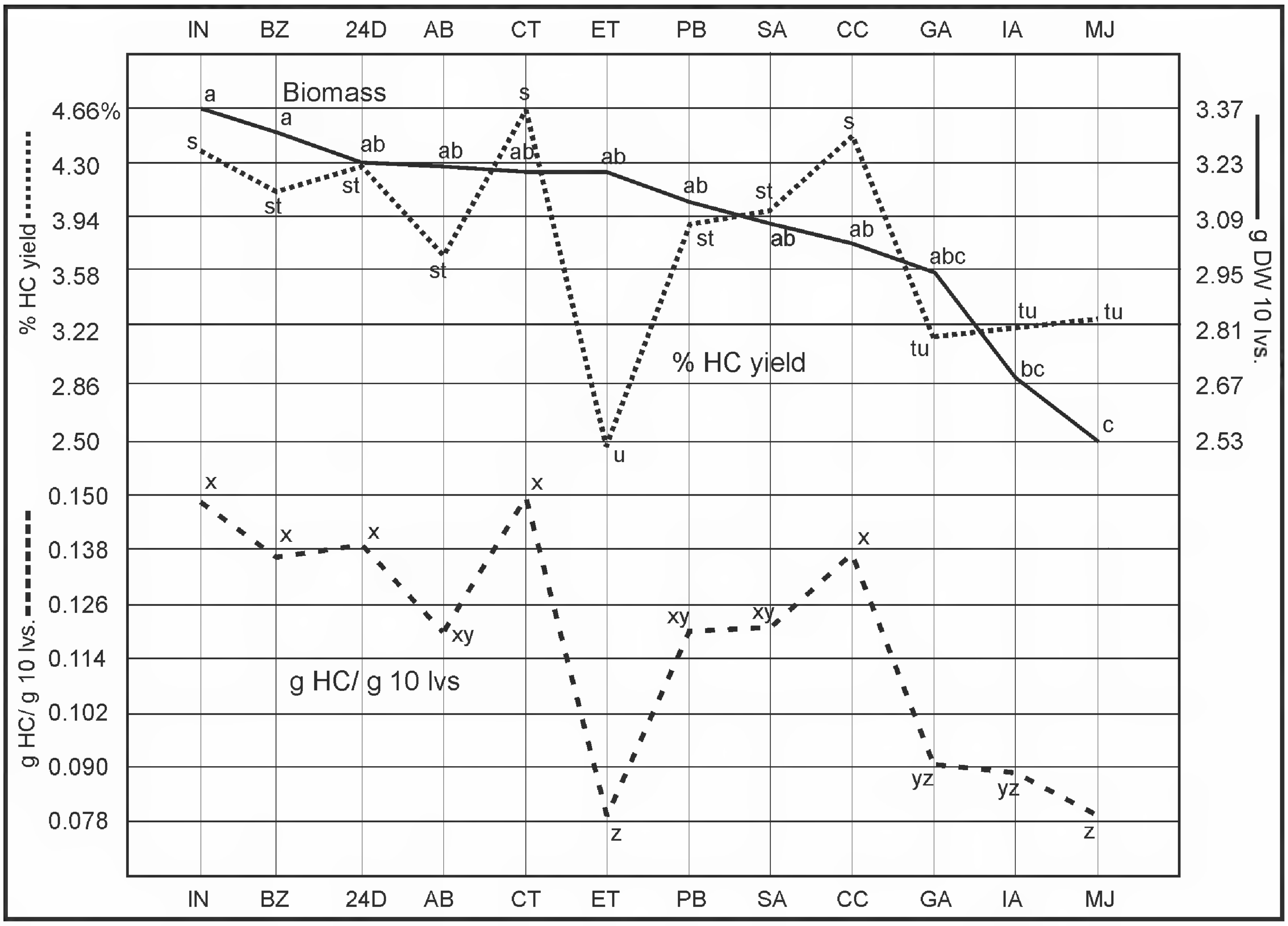

Figure 2. Graphs of dry weight (10 leaves), percent HC yields, and g HC/ gDW 10 leaves for Munchkin subjected to 12 treatments and analyzed 4 days later. Means with the same letter superscripts are not significantly different (P= 0.05). See text for discussion.

This research was initiated in the hope of stimulating the production of free HC for use as fuels from sunflowers. However, none of these treatments increased the production of HC in 4 days in cv. Munchkin. This may be due to the mixture of chemical classes such as terpenoids, lipids, waxes and sterols that are controlled by genes in various conflicting pathways. However, it should be noted that sunflowers growing in harsh, ambient conditions did produce very, much higher yields of HC than when grown in the greenhouse (Fig. 1). So, it still appears that some exogenous factor induces increase HC yields in sunflowers, although we did not discover the factor(s) in this study.

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The genus *Drosera* L. (Droseraceae) in the western USA

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ABSTRACT

The genus *Drosera* is well known among botanists and naturalists because of its carnivorous habit. In the USA most *Drosera* species occur in the eastern states. Well-known native and introduced taxa in the western USA, treated and keyed in this paper, include *D. anglica*, *D. capensis* (introduced), *D. linearis*, *D. rotundifolia*, and *D.* × *obovata*. *Drosera* × *woodii*, a sterile hybrid observed from a limited population in Montana during the course of this study, represents a previously unreported taxon for the Pacific Northwest. *Drosera intermedia*, widespread in the Eastern USA, but unknown for the western states, is also discussed because of previous, incorrect reports of it in the region. Additional non-native *Drosera* species are included in the discussion as appropriate.

In the western USA *Drosera* populations are often widely separated. In some cases, isolated populations have been identified as potentially rare occurrences meriting particular conservation efforts. Populations of potentially rare *Drosera* in the western states were visited in the course of this study, to review their identifications. Published on-line www.phytologia.org *Phytologia 101(1): 25-37 (March 21, 2019)*. ISSN 030319430.
KEY WORDS: *Drosera,* Droseraceae*,* western USA, carnivorous plants.

INTRODUCTION

The genus *Drosera* has more than 243 species worldwide (Lowrie *et al.* 2017), with 8 species currently recognized in North America (Rice *et al.* 2017). In the western states (MT, WY, CO, NM; and westward—that is, west of 105°W longitude) there are 3 species, mostly concentrated in the Pacific Northwest, and tending to occur in widely disjunct populations because of their restrictive habitat requirements. Populations occur from just a few meters above sea level to 2900 m. Because of site-to-site variations in habitat conditions, and possibly also because of genetic reasons, the phenotypic variation within a species can be significant. This has caused inconsistent identifications by field workers and taxonomists. In some cases, this has resulted in the (incorrect) reports of rare occurrences—this can and has resulted in conservation resources being applied to protect what were mistakenly thought to be rare elements (J. Costich-Thompson, L. Kinter, pers. comm. 2017). This paper serves as a comprehensive treatment of the genus in the western states to clarify these issues.

The native *Drosera* species of the western states are belong to the subgenus *Drosera*, section *Drosera* (Lowrie *et al.* 2017, Rice *et al.* 2017), which is a large but fairly natural group in the genus. The species in this region are restricted to full sun and perennially moist habitats (usually acidic or circumneutral pH) with low nutrient levels (Lowrie *et al.* 2017, Rice *et al.* 2017). Generally small perennials, they cannot survive long periods of inundation, nor can their weak root systems resist the rapid stream flows that may occur in riparian habitats during spring snowmelts. Gentle mountain seepages are ideal. The edges of lakes and ponds are suitable only if they maintain a constant water level. Floating vegetation mats (usually built upon a matrix based on *Sphagnum* or sedges) and floating, decaying logs are often excellent habitats.

In the eastern states, bogs and savannahs are often the preferred habitat of *Drosera*, but such habitats are rare in the west.

The yearly life cycle of all the native western *Drosera* species are similar. In the spring, the first leaves to emerge from the center of the rosettes tend to be small, and are not particularly helpful in identifying the species. Later leaves are larger and display species characteristics. The leaves are

petiolate, with long, sometimes flattened, mostly (but not always) glabrous petioles. The leaf blades are flattened, and the adaxial surfaces are covered with long, glandular hairs tipped with mucilage. At a distance, the plants can appear light green to deep red, and this overall color is due to contributions from leaf petiole, blade and the glandular tentacles. The degree of pigmentation is strongly affected by environmental effects (i.e., temporary inundation due to minor flooding, sun exposure, etc.), but occasionally very green populations may be encountered that may be governed by genetic factors (pers. obs.). Some populations can be so deeply red and extensive that they can be easily observed on satellite imagery.

Mature plants produce one or more inflorescences—erect, wiry, scorpioid racemes or weakly branching panicles. While flowering typically occurs in the spring, this depends on the site or even microsite; in sufficiently cold, harsh climates plants may be found flowering as late as September. Each day, one new flower opens in the morning, then closes by early afternoon. Little has been published about insects responsible for pollinating North American *Drosera* (Schnell 2002). Studying New Zealand *Drosera* species with similar floral and vegetative morphology, El-Sayad *et al.* (2016) observed pollination by a variety of *Diptera* (Syrphidae, Tachinidae, and Muscidae). Cultivated plants grown without the presence of pollinators also produce viable seed (pers. obs.).

Within several days after pollination, the fruit enlarge and if squeezed, will exude white, transparent immature seeds that can easily be seen with a hand lens. Mature seeds are black, grain-like, and have no apparent adaptations for dispersal, although their seed shapes and coats are distinctive and (at least in the western states) can be used to identify the species (Wynn, 1944). Hybrids occur in the western states, but these are sterile—after flowering, their fruit never enlarge, and instead remain slender.

Late in the season, leaves are produced that have petioles which are increasingly short and stout. The leaf blades are also reduced or absent entirely. In this manner, the plant transforms itself into a tight resting hibernaculum, by which the plant survives the winter cold and possible associated desiccation. Hibernacula of large plants are often multicrowned. In the spring, such multicrowned hibernacula often fragment, allowing for vegetative reproduction. This form of proliferation is the primary method of reproduction for sterile hybrids.

Seeds usually germinate in the spring, after a winter dormancy. Seedlings of all the species in our range are similar, and cannot reliably be distinguished. Often fruit do not fragment, but simply fall or are trampled into the soil—this can result in dense clusters of seedlings the following season.

For reference, the author maintains many carefully curated photographs of the taxa discussed in this paper, which can be reviewed at the CalPhotos web site (calphotos.berkeley.edu).

The carnivorous nature of *Drosera* is well established and described fully elsewhere (Lloyd 1942; Lowrie *et al*. 2017; Rice 2006; Schnell 2002; and others). Even so, it is remarkable, even to seasoned field scientists, how these organisms can capture surprisingly large prey such as dragonflies. This extraordinary behavior is also marvelously effective at capturing the interest of children, support by conservation donors, and enthusiasm by the general public.

Most of the *Drosera* species native to the USA are readily grown by the specialist, as long as their peculiar cultivation requirements are met (D'Amato 2013; Rice 2006). The primary cultivation challenge is associated with successfully providing an appropriate winter dormancy—during this period the winter hibernacula should be kept cold and moist. The length of the dormancy period, and the optimal temperatures, seems to depend upon the taxon and provenance, and can range from 3 to 7 months (pers. obs.). Most of these species are readily available from specialty nurseries, and in nearly all cases should not be field collected for cultivation.

The description above applies primarily to native species. Introduced exotics may differ, as noted in the discussions below.

The conservation status of *Drosera* in the western USA is currently fairly secure (Clarke *et al*. 2018). Most species occur over a wide range, and are often afforded a certain amount of protection as they are found in high quality wetlands (which have a suite of protective regulations). However, since they often occur in very small and isolated populations, they are prone to site-level extinction if there are significant changes in hydrology (either water flow or quality) or succession. Poaching is an annoyance, but *Drosera*

populations are usually limited by available habitat, and not reproductive potential—impact to sensitive habitat by trampling usually has more impact on populations than direct removal of plants.

METHODS

The following key uses a number of characters, so that plants even from aberrant populations can be confidently identified. It is best applied with mature, flowering or fruiting plants. Seedlings or young plants are not readily keyed out. Furthermore, even mature plants may not be readily identifiable early in the season as they emerge from hibernacula. While floral characteristics cannot be used to identify our native species, the seeds can be used definitively. In this key, the "leaf blade" is identified as the laterally expanded portion of the leaf that bears long, mucus-tipped glandular hairs; i.e., not including the non-glandular petiole. Seeds are easily found in immature and mature fruit, and can often even be found intact in fruit from previous seasons.

Specimens collected by the author (under permit, as appropriate) are stored at DAV.

Key to the *Drosera* in the western USA

1. Forming an erect stem; roots thick (diameter > 1 mm) and fleshy; inflorescence scape more than 1 mm in diameter and densely hairy; flowers large (> 15 mm across) with usually pale purple petals, rarely white; plants never forming tight winter resting buds……………*Drosera capensis* [Introduced exotic]
1. Leaves all from a basal rosette, forming an elongate stem only in extremely etiolated conditions; roots threadlike (diameter < 0.5 mm); inflorescence threadlike and less 0.5 mm in diameter and not hairy; flowers small (< 9 mm across) with white petals; plants produce tight hibernacula during the winter.
   2. Flowering-sized plants with leaf blades that are as wide, or wider, than long; leaves—except for the most recently produced—nearly all appressed against the ground in a flat rosette ………………………………………………………………………………………*Drosera rotundifolia*
   2. Flowering-sized plants with leaf blades that are longer than wide; leaves nearly all vertical or held at various angles, but mostly upwards.
      3. Leaf shape linear—leaf blade margins parallel over most of the leaf blade length; seeds rhomboidal, crateriform, 0.5-0.8 mm long; leaf blade length 5-8(20) × leaf blade width ………………………………………………………………………………………*Drosera linearis*
      3. Leaf shape short to long oblanceolate—leaf blade margins slowly widening from base for 2/3 or more of leaf blade length, then converging to blunt tip; seeds absent or if present, then fusiform, areolate-striate, 1-1.5 mm long; leaf blade length 1.3-5(15) × leaf blade width.
         4. In fruit, once approximately five flowers have opened, the lowest flowers will contain white to black grain-like seeds easily seen at 10×; leaf blade reaches maximum blade width approximately 2/3 the distance from blade base, then narrows to blunt tip; leaf blades generally more elongate than those of any *Drosera* hybrids present ………………………………………………………………………………*Drosera anglica*
         4. Fruit never produce seed; leaf blade continuously widens along leaf blade length, reaching maximum width nearly at the blunt leaf tip; leaf blades generally less elongate than those of *D. anglica* or *D. linearis* (if either are present) ……………………………………………………………Hybrids: *Drosera* × *obovata, D.* × *woodii*

As described in the DISCUSSION section below, *D. intermedia* has been incorrectly reported in extreme northern Idaho (Bursik 1993). This report has led to speculation it might occur elsewhere in the state. Reliable characteristics that can be used to separate *D. intermedia* from western USA species are given in the following key.

Key to *Drosera intermedia* vs. western USA *Drosera* species

1. Seed coat uniformly papillose; inflorescence emerges from the rosette center nearly horizontally, then arcs upwards to one side of the plant; flowering-sized plants with leaves held at various angles—from nearly straight up to horizontally, so that overall the plant occupies a hemispherical volume ………………………………………………………………………………………….*Drosera intermedia*
1. Seed coat minutely rough or areolate-striate, or mature seeds absent; inflorescence emerges vertically (or nearly so) from the rosette center; flowering-sized plants with leaves that (except for the most recently produced) are nearly all appressed against the ground in a flat rosette (*D. rotundifolia*) OR the leaves on flowering-sized plants are held mostly vertically, so that overall the plant occupies a volume that is cylindrical, taller than wide (all other taxa) ……………………………..*Drosera anglica, D. linearis, D. rotundifolia, D. x obovata, D. x woodii*

DISCUSSION

***Drosera rotundifolia* L.**

Key features: Flat rosette; leaf blades wider than long.

Western States reported: CA, OR, WA, MT, ID, only 4 verified sites in CO (Rice *et al*. 2017; Wolf *et al*. 2006).

Global range: A circumboreal species; outlying populations in the Philippines and New Guinea.

Habitats: Throughout its range, a surprisingly adaptable species. Open seepages—ranging from nearly level to steeply sloped—with little shading or competition are typical (Rice *et al*. 2017; Rice 2006; Schnell 2002). It can also be found in peculiar habitats such as floating *Sphagnum* mats or rotting logs, sheer rock surfaces covered with films of water, serpentinite soils (with *Darlingtonia californica*), and even geothermal fens (pers. obs.).

Comments: See Figure 1. Usually a relatively small species that may be only 3 cm across at flowering. In ideal situations, especially when extremely wet, plants can have much elongated petioles that result in a rangy, fragile architecture; such plants may be 10 cm across or more. Seeds are several times longer than wide, with acute tips, and longitudinally striate (see Figure 10).

This is the most abundant of the *Drosera* species in the western USA, occurring in the broadest range of habitats. However, it is perplexing that in large portions of the west, wetland habitats support *D. rotundifolia* and very rarely *D. anglica*, while in other portions of the west, the same habitats commonly support *D. anglica* and very rarely *D. rotundifolia*.

This species is easily cultivated by specialists.

***Drosera anglica* Huds.**

Key features: Vertically oriented leaves; leaf blades narrowly oblanceolate.

Western States reported: CA, OR, WA, MT, ID, WY, 1 site in CO (Rice *et al*. 2017; Wolf *et al*. 2006).

Global range: A circumboreal species.

Habitats: In California and Oregon, usually found only on *Sphagnum* mats and only rarely on open seepages. Elsewhere in the western USA it is more common in grassy or sedgy seepages, or on open flats of organic muck.

Comments: See Figures 2, 3. Quite variable in size and in leaf shape. In some regions (especially Idaho), the plant may be relatively prostrate and only 2-3 cm in diameter, with leaf blades only 1.3× longer than wide, encouraging misidentifications with *D. rotundifolia* (see also comments in *D. intermedia*, below). Hybrids between such small *D. anglica* with *D. rotundifolia* would be potentially very difficult to detect, unless particular attention were paid to infructescenses (fertile: *D. anglica*, sterile: *D. × obovata*). In some locations (especially California), plants have extremely long, almost linear leaves that can be approximately 15× longer than wide. Even in such elongate specimens, the leaf blades slowly widen from the petiole width to the maximum leaf blade width as described in the key couplet #4.

Seeds are fusiform, and covered with tiny windows arranged in rows (see Figure 10).

It is widely believed that *D. anglica* originated long ago as a hybrid between *D. linearis* and *D. rotundifolia* (both 2N=20). Indeed, in Michigan where the ranges of two parental species overlap in Michigan, the hybrid *D. linearis* × *rotundifolia* (= *D.* × *woodii*) has been detected, and is almost indistinguishable from *D. anglica*, except for being sterile. This hybrid is believed to have subsequently become fertile via a natural chromosome doubling event. The new, fertile entity spread from its point of origin throughout the northern hemisphere, as the highly successful modern species *D. anglica* (2N = 40) (Rice *et al.* 2017; Schnell 2002; Wood 1955).

This species is relatively easy to grow for specialist horticulturists, although it is rare for cultivated plants to be as large as may be observed in the wild. Specimens from populations marked by small, short leaved plants (Valley County, ID; Boundary County, ID), were grown side-by-side with specimens from populations with large, elongate leaves (Butte County, CA). Despite being grown by an expert horticulturist, the plants from the Idaho populations were comparatively difficult to maintain in cultivation, but pilot results suggest that they did maintain dwarfed characteristics in cultivation (Collingsworth, D., pers. comm. 2018, 2019).

***Drosera linearis* Goldie**

Key features: Vertically oriented leaves; leaf blades linear.

Western States reported: Three localized clusters of remote sites in MT (Rice *et al.* 2017; Montana Natural Heritage Program, pers. comm. 2017; Hitchcock & Cronquist, 2018). Reports of a population in Lake County, MT are erroneous—visits to the site have detected *D. rotundifolia*, but the site was otherwise inconsistent with *D. linearis* habitat (pers. obs.).

Global range: USA (MN, WI, MI, ME, MT), Canada (AB, ON, QC, NB, NL; possibly also BC, MB, NT, SK).

Habitats: Highly specialized, occurring in the muck on floating sedge-dominated vegetation mats or lake margins. In the western USA, only in remote mountain fens.

Comments: See Figure 4. This plant can bear a superficial resemblance to very long-leaved strains of *D. anglica*, but can reliably be distinguished by leaf blade outline, or seed characters, as noted in the key.

This species is primarily found in the Great Lakes area, and eastward. The Montana populations are remarkably remote. However, a few sites are also found to the north in British Columbia (Jenifer Penny, pers. comm. 2016), Manitoba (Chris Friesen, pers. comm. 2016), and Saskatchewan (Beryl Wait, pers. comm. 2016). Broadly speaking, these bridge the Montana plants to the eastern populations.

This species is extremely difficult to cultivate, even for experts (D'Amato 2013), and cultivated plants usually perish after a few years. While horticulturists have been known to introduce carnivorous plants to sites outside their native ranges (see below), it is extremely unlikely that the plants in Montana represent artificially introduced plants. The sites are remote—some taking days of pack travel to reach—in habitat frequented by grizzly and black bears. Meanwhile, locations targeted by people attempting introduction experiments are typically roadside or otherwise easily accessible.

***Drosera* × *obovata* Mert. & Koch (=*D. rotundifolia* × *anglica*)**

Key features: Vertically oriented leaves; leaf blades obovate; fruit always sterile.

Western States reported: CA, OR, 1 site in WA (Rice *et al.* 2017; WTU specimen 314866; Hitchcock & Cronquist, 2018).

Global range: Sporadically detected wherever both parent species are found near each other.

Habitats: Usually *Sphagnum* dominated flats, occasionally other wet seepages.

Comments: See Figure 5. To be expected whenever the parent species *D. anglica* and *D. rotundifolia* occur in close proximity, but occasionally found at sites with one of the parent species absent. (Why one of the parents may be absent is not known—either a pollinator may have introduced pollen of the absent species, or some stress may have resulted in the local extinction of one of the parent species.) *D.* × *obovata* may occur (as yet undetected) in ID and MT, and possibly even CO.

Late season plants can easily be distinguished from *D. anglica* by the lack of seeds. In *D.* × *obovata*, the old, dried flowers are slender and lack seeds; in *D. anglica*, the fruit develop soft white, then hard

black seeds within a week of flowering—these seeds are retained in the fruit throughout the rest of the growing season.

This hybrid generally has leaf characteristics intermediate between the parent species. In California and Oregon sites, where the parent *D. anglica* may have extremely long leaves, *D.* × *obovata* can have leaf blades 3-3.5 × longer than wide. In regions where the parent *D. anglica* has much shorter leaves (such as Idaho), *D.* × *obovata* might only be reliably detectable by the lack of seeds. This suggests that *D.* × *obovata* is probably generated locally, and does not spread readily. This is understandable for a hybrid plant that only propagates itself vegetatively. However, this could be explored in greater detail in common greenhouse cultivation.

Drosera × *obovata* is easily grown by horticulturists. Extremely large specimens from Butte County, CA that were collected in the course of the research for this study, were grown in cultivation and retained their oversized attributes (Collingsworth, pers. comm. 2018, 2019).

***Drosera* × *woodii* Gauthier & Gervais (= *D. rotundifolia* × *linearis*)**

Key features: Vertically oriented leaves; leaf blades obovate; fruit always sterile.

Western States reported: 1 site in MT.

Global range: Known only from MI and MT.

Habitats: In the western USA, known from only one sedge-dominated fen; only in the muck on detached, floating vegetation mats.

Comments: See Figures 6, 7. During the course of research leading to this paper, a Montana site reportedly supporting *D. linearis* and *D. anglica* was investigated. While the *D. linearis* was verified, the "*D. anglica*" was found to be a heterogenous population of short and long leaved, sterile hybrids. Collections of this population made in 1985 (MONTU 102206, 102480) were incorrectly identified as *D. anglica*. This is the first report of (putative) *D.* × *woodii* from the western USA.

The identity of the short-leaved and long-leaved hybrid populations cannot be determined with complete certainty. *Drosera linearis* is present and is probably one of the parent plants. True *D. anglica* was not found at the site. *Drosera rotundifolia* was reported in the immediate area in 2004 (Montana Natural Heritage, 2017), but no herbarium collections were made. *Drosera rotundifolia* was not found on two visits in 2017 conducted during the course of this study. It is possible that the reports of *D. rotundifolia* are due to observations of short-leafed hybrid individuals. The morphology of the hybrids, especially plants in the short-leaved population, strongly suggest one of the parent plants was *D. rotundifolia*. In this paper I tentatively conclude that the two hybrid populations are best treated as *D.* × *woodii*. It seems unlikely, but it is possible the hybrid plants are actually *D. linearis* × *anglica* (= *D.* × *linglica* Kusakabe ex Gauthier & Gervais).

Both *D.* × *woodii* and *D.* × *linglica* have been found only in Michigan (Schnell 2002) and Quebec (Brouillet *et. al.*, 2010). In Michigan, they only occur at sites where both parent species are found (Schnell 2002). The presence of *D.* × *woodii*, in two populations (both short-leafed and long-leafed plants) is a double-rarity for this extremely interesting site.

A complete census of the site was not conducted, however rapid visual assessments indicated that the population of both short-leaved and long-leaved plants ranged from several hundred, to thousands, or even more. Based upon horticultural experience with sterile *Drosera* hybrids, these plants probably proliferate by the production of secondary, lateral hibernacula in dormancy (which can easily detach).

This hybrid is not in cultivation, and as such the site is subject to potential conservation impacts from collection. However, this site is large enough, and so remote, that casual visitation is unlikely to cause significant damage. Repeat visitation is more likely to cause significant trampling damage, and such impacts should be taken into consideration if any research plans are proposed.

***Drosera capensis* L.**

Key features: Stem-forming, roots thick (diameter > 1 mm) and fleshy, inflorescence scape thick (diameter > 1 mm) and densely hairy; flowers large and usually lilac to purple.

Western States reported: CA: Mendocino County, Del Norte County (Rice 2002; pers. obs.).

Global range: Native to South Africa, but may be encountered elsewhere as a non-native species introduced by horticulturists.
Habitats: Wet seepages.
Comments: See Figure 8. This commonly cultivated African species (Robinson *et al*. 2017) is the most frequently encountered non-native *Drosera* in the western USA. Plants less than a year old are similar in form to young *D. anglica* or *D. linearis*, although the subsucculent roots are much thicker than the fibrous roots of the native species. The flowers are usually pink to purple (very rarely white), and are on thick, hairy inflorescences. Hibernacula are not produced.

In Mendocino County (CA), *D. capensis* is certainly an intractable weed at one site (Rice 2002). It is occasionally encountered in Del Norte County (CA), where horticulturists have repeatedly introduced plants into *Darlingtonia* seepages (pers. obs.). This is the origin of reports of *D. linearis* in Del Norte County (Stone 1993).

Plants are usually top-killed by freezing conditions, but can readily regenerate from seeds or root systems. Populations are likely to be detected in coastal OR, WA, and BC (Canada).

***Drosera intermedia* Hayne**
Key features: Leaves oriented so as to describe a spherical volume; scape horizontal at base; seed coat papillose.
Western States reported: ID (Bursik 1993), but incorrectly.
Global range: Europe; eastern Canada; eastern half of USA, south to northern South America.
Habitats: Wet seepages.
Comments: See Figures 9, 10. *Drosera intermedia* is a species with wide distribution in eastern North America, and globally in Latin America and Europe (Rice 2006). Its supposed presence in Idaho dates to reports by Bursik (1993). In this paper, Bursik noted the presence of *D. intermedia* in Smith Creek RNA (Boundary County, ID). This promulgated additional reports in the Sawtooth Mountains in Custer County, ID (L. Kinter, pers. comm. 2017). As part of this study, Smith Creek RNA was surveyed, and the purported "*D. intermedia*" plants were refound. Their growth forms, leaf and seed characteristics were all consistent with *D. anglica*. The plants at this site have an interesting clustering character, but are otherwise typical. Plants from these sites have proven to be surprisingly difficult to grow in greenhouse cultivation, even by expert horticulturists (Collingsworth, pers. comm. 2018, 2019). It is still not clear if the interesting, clumping character is genetic or is due to environmental conditions. The Custer County, ID sites were also surveyed, and the plants there were similar entirely consistent with *D. anglica*.

The papillose seed coats of *D. intermedia* are diagnostic, and visible at 10× (see Figure 10). None of the plants in the western USA had such seeds.

There is no remaining evidence for native *D. intermedia* in the western USA. However, skeptical field workers finding suspected "*D. intermedia*" should examine the "Key to *Drosera intermedia* vs. western USA *Drosera* species," given above.

Exotic *Drosera* species of extremely limited range

Carnivorous plant horticulturists have had a long and unfortunate interest in planting non-native carnivorous plants in the wild. In Mendocino County (CA), horticulturists have been introducing non-native carnivorous plants from many genera to a wildland location owned by The Nature Conservancy. Despite Conservancy staff attempts to stop this activity by outreach to horticulturists, and by some removal activity, more than twenty non-native carnivorous plant taxa have been found on the site (Rice 2002). In addition to *D. capensis*, *Drosera* observed at this site include *D. aliciae*, *D. binata, D. burmanni, D. capillaris, D. filiformis, D. intermedia, D. slackii,* and others (pers. obs.).

Another plant—probably *D.* × *hybrida* Macf., but possibly an allied hybrid—has been detected at Butterfly Valley Botanical Area (Plumas County, CA), and after a few years of work has been extirpated (Rice 2005). This is the origin of reports of *D. anglica* in the Butterfly Valley watershed, as recorded in unpublished US Forest Service plant lists for the region (ca. 1992).

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Figure 1: *Drosera rotundifolia.* Plants as typically seen (Tehama County, California). Note the ground-hugging habit, and leaves with glandular blades wider than long. Additional images can be seen at https://calphotos.berkeley.edu/ by selecting "Scientific Name EQUALS" *Drosera rotundifolia*, and "Photographer EQUALS" Barry Rice.

Figure 2: *Drosera anglica.* Various forms of this plant—Top-left: long-leaved plants (Plumas County, California); Top-right: typical, medium-leaved plants (Butte County, California); Bottom-left: short-leaved plants (Valley County, Idaho); Bottom-right: very green plants (Custer County, Idaho). Additional images can be seen at https://calphotos.berkeley.edu/ by selecting "Scientific Name EQUALS" *Drosera anglica*, and "Photographer EQUALS" Barry Rice.

Figure 3: *Drosera anglica.* Short-leaved, clumping plants that have been incorrectly identified as *D. intermedia* (Boundary County, Idaho).

Figure 4: *Drosera linearis.* Typical plants (Lewis and Clark County, Montana). Note how the margins of the leaf blades are parallel over much of their lengths. Additional images can be seen at https://calphotos.berkeley.edu/ by selecting "Scientific Name EQUALS" *Drosera linearis*, and "Photographer EQUALS" Barry Rice.

Figure 5: *Drosera x obovata.* Plants with very typical leaf shape (Butte County, California). Additional images can be seen at https://calphotos.berkeley.edu/ by selecting "Scientific Name EQUALS" *Drosera x obovata*, and "Photographer EQUALS" Barry Rice.

Figure 6: *Drosera x woodii.* The long-leaved form of this plant is at center and left, while *Drosera linearis* is at right (Lewis and Clark County, Montana). Notice the slender seedpods on the tall inflorescences of the sterile hybrid, in contrast with the plump seedpods on the short inflorescences of *Drosera linearis* visible here and in Figure 4.

Figure 7: *Drosera x woodii.* The short-leaved form of this plant (Lewis and Clark County, Montana). The overall plant morphology is strongly suggestive of a *Drosera rotundifolia* parentage.

Figure 8: *Drosera capensis.* The morphology of the leaf-blade is similar to *Drosera linearis* (Del Norte County, California). *Drosera rotundifolia* is also visible in the rear left. Additional images can be seen at https://calphotos.berkeley.edu/ by selecting "Scientific Name EQUALS" *Drosera capensis*, and "Photographer EQUALS" Barry Rice.

Figure 9: *Drosera intermedia.* Comparison images for this species, which is not found in the western USA—Left: notice how the scape emerges from the rosette nearly horizontally, then curves upwards (Left: Pender County, North Carolina); Right: a stem-forming individual (Columbus County, North Carolina). Notice that in both plants, leaves are held at a variety of angles, so the upper portion of the plant occupies a hemispherical region. Additional images can be seen at https://calphotos.berkeley.edu/ by selecting "Scientific Name EQUALS" *Drosera intermedia*, and "Photographer EQUALS" Barry Rice.

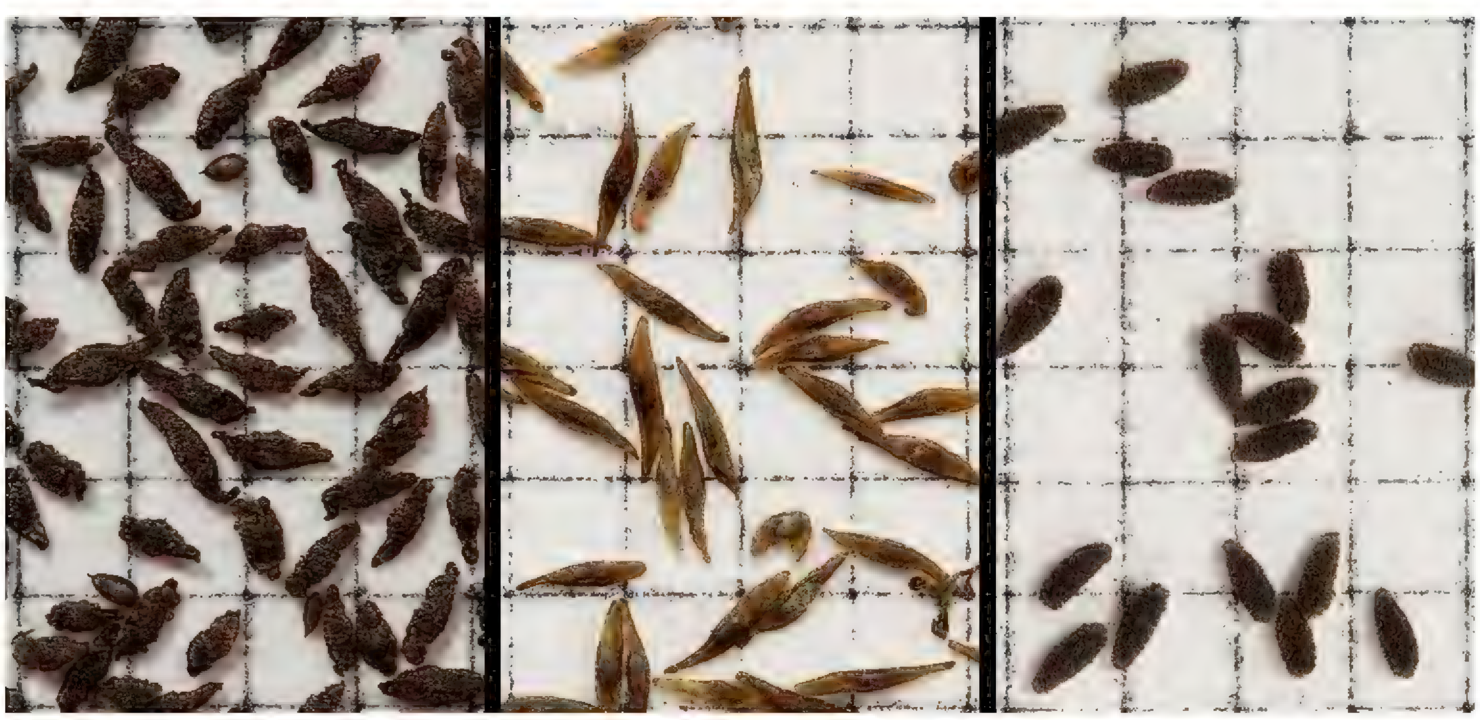

Figure 10: Seed coats of *D. anglica*, *D. rotundifolia*, and *D. intermedia*, photographed on a 1 mm grid. Note the papillose seed coats of *D. intermedia*. The seeds of *D. linearis* are not shown, but do not bear papillae. Seed photographs provided by John Brittnacher.

The composition of the leaf essential oils of *J. sabina* var. *balkanensis*: comparison between oils from central Italy with oils from Bulgaria, Greece and Turkey

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Abstract

The composition of the leaf oil of *J. sabina* var. *balkanensis* from central Italy was compared to volatile leaf oils from Bulgaria, Greece and Turkey, as well as *J. sabina* var. *sabina* from Switzerland, Pyrenees, and Kazakhstan. The leaf oils in central Italy had chemotypes with some plants of *J. sabina* var. *balkanensis* oils with high sabinene (HiSab, 28.4 - 40.2%), low trans-sabinyl acetate chemotype (LoTSac) and other plants with the low sabinene (LoSab, 9.6 - 14.8%), high trans-sabinyl acetate (HiTSac, 36.0 - 47.4%) chemotype. These same chemotype patterns were also found in Bulgaria, Greece, and Turkey. There appear to be no consistent chemical differences between the oils of var. *balkanensis* in Bulgaria, Greece, Turkey and the oil from central Italy. The lack of chemical differentiation between eastern (Bulgaria, Greece, Turkey) and western (Italy) var. *balkanensis* populations may reflect the recent origin of var. *balkanensis* or gene flow. Comparing the compositions of the leaf essential oils of central Italy (var. *balkanensis*) with oils of *J. sabina* var. *sabina*, from Pyrenees, and Switzerland revealed oils of *J. s.* var. *balkanensis* differ only slightly from *J. s.* var. *sabina*, Switzerland and Pyrenees. The presence of chemotypes in var. *balkanensis* populations make it very difficult to determine any differences among var. *balkanensis* populations and between var. *balkanensis* and var. *sabina* oils. Published on-line www.phytologia.org *Phytologia 101(1): 38-45 (March 21, 2019)*. ISSN 030319430.
KEY WORDS: *Juniperus sabina* var. *balkanensis*, volatile leaf oils, terpenes, composition. *J. sabina.*

As part of an on-going investigation of *Juniperus sabina* L., we investigated the leaf volatile oil composition of var. *balkanensis* R. P. Adams & A. N. Tashev from central Italy, where it was recently discovered (Adams, et al. 2018a). *Juniperus s.* var. *balkanensis* was initially discovered in Bulgaria (see Adams, Schwarzbach and Tashev, 2016).

Juniperus sabina var. *balkanensis* contains the chloroplast of *J. thurifera* and nuclear nrDNA of *J. sabina* L. (Adams, Schwarzbach and Tashev, 2016). The new variety appears to be morphologically,

nearly identical to *J. s.* var. *sabina.* In addition to the type locality in Bulgaria, Adams et al. (2017) later reported var. *balkanensis* in far western Turkey and in northern Greece, and as far west as Macedonia, Croatia and central Italy. (Adams et al. 2018a).

The volatile leaf oils of *J. sabina* have been often analyzed (see a review in Adams, Nguyen and Liu, 2006). In same paper they reported on a detailed analysis of the composition of the leaf oils from seven populations of *J. sabina* and one population of *Juniperus sabina* var. *arenaria* (E. H. Wilson) Farjon (now treated as *J. davurica* var. *arenaria* (E. H. Wilson) R. P. Adams), as well as the oils of *J. chinensis* L. and *J. davurica* Pall. Adams, Nguyen and Liu (2006) found considerable differentiation in populations of *J. sabina* from the Iberian Peninsula and far eastern populations (Kazakhstan, China). The amounts of cedrol, citronellol, safrole, trans-sabinyl acetate, terpinen-4-ol and trans-thujone were found to be polymorphic in several populations.

The leaf oils of the aforementioned species, except var. *balkanensis*, have been reported, and reviewed: *J. chinensis* (Adams, Chu and Zhong, 1994); *J. davurica* (Adams, Shatar and Dembitsky, 1994), *J. sabina* (Adams, Dembitsky and Shatar, 1998, Adams, 2014).

Analyses of the volatile leaf oils of var. *balkanensis* from Bulgaria, Greece and Turkey (Adams et al. 2018b) revealed considerable polymorphisms in the oils, notably in high amounts of trans-sabinyl acetate (HiTSac) with low amounts of sabinene (LoSab) and vice versa (Table 1). All plants sampled in the Greece population were low trans-sabinyl acetate (LoTSac)/ high sabinene (HiSab) (Table 1). However, both the Bulgaria populations (eastern Rhodopes and Rilla Mtns.) contained plants with HiTSac/LoSab and LoTSac/ HiSab. Interestingly, the *J. sabina* var. *sabina* oils from Switzerland (SWZ), Pyrenees (PYR) and Kazakhstan (KAZ) were all high in sabinene (HiSab), but the oil of SWZ was also a HiTSac population, whereas PYR and KAZ had LoTSac oils (Table 1). Several plants had high concentrations of individual components: α-pinene, cis-thujone, trans-thujone, trans-sabinol, methyl eugenol and elemicin (highlighted in green in Table 1). As reported by Adams, Nguyen and Liu (2006), components typical of *Juniperus* wood oils (α-cedrene, allo-cedrol, cedrol, epi-cedrol, α-acorenol) were found only in the oil from Kazakhstan. The presence of several chemotypes precluded analysis of any trends among the oils of the populations.

The purpose of this paper is too extend analyses on the composition of the volatile leaf oil(s) of *J. sabina* var. *balkanensis* to the western-most known populations in central Italy.

MATERIALS AND METHODS

Specimens used in this study (species, popn. id., location, collection numbers):
J. sabina var. *balkanensis*
Bulgaria and Greece
BeR: Eastern Rhodopes. In protected site "Gumurdjinsky Snejnik", locality "Madzharsky Kidik". On limestone rocks above the upper border of a forest of *Fagus sylvatica* ssp. *moesiaca* with *Juniperus communis*. 41° 14' 44.7" N; 25° 15' 31.9" E. elev. 1270 m, 13 Aug. 2012, *Adams 13725-13729 (A. Tashev 2012-1-5)*;
BSk: Central Stara Planina (the Balkan). National Park "Central Balkan". Reserve "Sokolna". On a steep, rocky limestone slope, with *Sorbus aucuparia, S. aria, S. borbasii, Amelanchier ovalis, Carpinus orientalis, Sesleria latifolia, Pastinaca hirsute, Cephalanthera rubra, Laserpitium siler, Hieracium alpicola* etc. near a forest of *Fagus sylvatica.* 42°42'13.3" N,25°08'10.4" E, 1501 m , 22.08.2015. Bulgaria, *Adams 14721 (A. Tashev 2015 Balkan 1*;
BkR: Rila Mountain, National Park "Rila". On the eco-path, Beli Iskar", near river Beli Iskar, in a forest with *Pinus sylvestris, P. peuce, Picea abies, Abies alba, Juniperus communis, J. sibirica, Vaccinium*

myrtillus, Rosa canina, Sorbus aucuparia, Acer hyrcanum, Chamaespartium sagittale, Hypericum perforatum, Thymus sp. etc. 42°14'26.5" N, 23°32'33.8" E, 1242 m, 24.06.2015. Bulgaria, *Adams 14722-14726 (A. Tashev 2015 Rila 1.1-1.3, 2.1-2.2)*;
BkG: Mt. Tsena, Greece, *Adams 14727-14731* (*A. Tashev 2015 So. 1-5 Tsena*);

Italy

Val di Foro, loc. Colle dell'Angelo, radura boschiva, Coll. Fabrizio Bartolucci, F. Conti, L. Di Martino 61-2082, A1, A2, 42.19372° N,14.12086° E, 1002 m,10 July 2018, Lab Acc. Robert P. Adams 15500, 15501; Colle le Macchie, Coll. Fabrizio Bartolucci, F. Conti, L. Di Martino 64-2245 B1,B2, 42.10842° N, 14.19584° E, 1030 m,10 July 2018, Lab Acc. Robert P. Adams 15502, 15503; M. San Domenico (Pizzoferrato, Chieti) rupi, 1484 m, Coll. Fabrizio Bartolucci, F. Conti, L. Di Martino 64-2241, C1,C2, 41.92854° N, 14.21135° E, 10 July 2018, Lab Acc. Robert P. Adams 15504, 15505.

Turkey

Bk/Turk: Spil Daği Milli Parki (National Park), Turkey, Manisa, 38°, 57' N, 27° 41' E, 1024 m. *Adams 14934*, (*Tuğrul Mataraci 2016-1*)

Other plants referenced in this paper:

J. chinensis, CH, Lanzhou, Gansu, China, Adams 6765-67; *J. davurica*, DV, 15 km se Ulan Bator, Mongolia, Adams 7252, 7253, 7601; *J. sabina*, SN, Sierra Nevada, Spain, Adams 7197, 7199, 7200; PY, Pyrenees Mtns., Spain/ France border, Adams 7573-77; SW, 2 km s of St. Niklaus, Switzerland, Adams 7611, 7612, 7614, 7615; KZ, 30 km n. of Jarkent, Kazakhstan, Adams 7811-13; AM, Altair Mtns., Mongolia, Adams 7585-88; TS, Tian Shan Mtns., Xinjiang, China, Adams 7836-38; MS, sand dunes, 80 km sw Ulan Bator, Mongolia, Adams 7254-56; AR, sand dunes, Lake Qinghai, Qinghai, China, Adams 10347-52.
Voucher specimens for all collections are deposited at Baylor University Herbarium (BAYLU).

Fresh, air dried leaves (50-100 g) were steam distilled for 2 h using a circulatory Clevenger-type apparatus (Adams, 1991). The oil samples were concentrated (ether trap removed) with nitrogen and the samples stored at 20 ºC until analyzed. The extracted leaves were oven dried (100 ºC, 48 h) for determination of oil yields.

Oils from 4-5 trees of each taxon were analyzed and average values reported. The oils were analyzed on a HP 5971 MSD mass spectrometer, scan time 1/ sec., directly coupled to a HP 5890 gas chromatograph, using a J & W DB-5, 0.26 mm x 30 m, 0.25 micron coating thickness, fused silica capillary column (see Adams, 2007 for operating details). Identifications were made by library searches of our volatile oil library (Adams, 2007), using the HP Chemstation library search routines, coupled with retention time data of authentic reference compounds. Quantitation was by FID on an HP 5890 gas chromatograph using a J & W DB-5, 0.26 mm x 30 m, 0.25 micron coating thickness, fused silica capillary column using the HP Chemstation software.

RESULTS

The compositions of the leaf oils are given in Table 2. For central Italy, three plants of *J. sabina* var. *balkanensis* oils were high sabinene (HiSab, 28.4 - 40.2%), low trans-sabinyl acetate (LoTSac) chemotypes and three were LoSab/ HiTSac chemotypes (Table 2). This pattern was also found in Bulgaria (Table 1, east. Rhod. and Rila).

Although there are no chemical polymorphisms shown (Table 1, 2), for the three *J. s.* var. *sabina* populations (SWZ, PRY, KAZ), oils from the Sierra Nevada, Spain area have been reported as the most polymorphic of all the var. *sabina* populations examined (Adams et al. 2006), with large variations for trans-thujone (0.5 - 8.7%), terpinen-4-ol (3.6 - 14.4%), trans-sabinyl acetate (6.4 - 41.3%), and methyl eugenol (0.01 - 12.1%). Additional samples from Spain are needed.

A few compounds, sabina ketone, (2E,4Z)-methyl decadienoate, (E)-caryophyllene, trans-murrola-3,5-diene, trans-cadina-1(6),4-diene and cubebol appear to be larger in the Italy samples than in the var. *sabina* populations (SWZ, PRY, KAZ, Table 2). However, most of these compounds are in low concentrations, so additional analyses may not support these small differences. It might be noted that our previous work (Table 1 above, updated from Adams, 2018b) gave a similar pattern. It is interesting to note that trans-muurola-4(14),5-diene was only a trace in the Bulgaria, Greece, Turkey populations (Table 1), but ranged trace to 1.7% in the central Italy plants (Table 2).

There seem no consistent chemical differences between the oils of var. *balkanensis* in Bulgaria, Greece, Turkey and the oil from central Italy. The lack of chemical differentiation between eastern (Bulgaria, Greece, Turkey) and western (Italy) populations may reflect the recent origin of var. *balkanensis,* considerable gene flow among populations, or only small amounts of natural selection.

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Table 1. Comparisons of the per cent total oil for leaf essential oils for *J. sabina* var. *balkanensis* and *J. s.* var. *sabina* populations. *J. s.* var. *sabina* population codes: PYR Pyrenees Mtns.; SWZ, Switzerland; KAZ, Kazakhstan; Components that tend to separate the species are highlighted. Modified from Adams et al. 2018b. See text for discussion.

		J. sabina var. *balkanensis*							*J. s.* var. *sabina*		
		low trans-sabinyl acetate (LoTSac), and high sabinene (HiSab)					high TSac, low sabinene		high TSac	low TSac	
RI	Compound	Tur-key	Greece		Bulg. east. Rhod	Bulg. Rila mts.	Rila Hi TSac	Rhod Hi TSac	SWZ Hi TSac	PYR Lo TSac	KAZ Lo TSac
931	α -thujene	1.2	1.2	1.7	1.3	1.0	0.3	0.5	0.9	1.0	0.6
939	α-pinene	2.4	3.7	2.6	3.0	1.3	2.2	1.3	2.0	1.8	15.8
953	camphene	t	-	t	t	t	t	t	t	t	0.3
976	sabinene	56.1	42.5	59.7	41.2	41.3	3.7	7.5	34.8	54.9	42.6
980	β-pinene	t	0.3	0.2	0.4	t	0.6	0.3	t	t	0.7
991	myrcene	3.4	2.8	3.9	2.9	2.7	1.6	2.0	4.2	3.1	3.8
1005	α-phellandrene	t	t	t	0.1	0.1	t	t	t	0.1	t
1011	δ-3-carene	0.1	1.5	t	0.1	0.1	0.1	1.0	-	0.1	0.2
1018	α-terpinene	1.2	1.4	1.6	1.6	1.6	0.3	0.4	1.0	1.5	0.7
1026	p-cymene	0.3	0.4	0.5	0.6	0.5	0.6	0.4	0.2	0.4	0.1
1031	limonene	1.5	0.8	1.3	0.6	1.0	0.8	0.6	3.0	2.4	2.1
1031	β-phellandrene	1.6	0.8	1.3	0.9	1.0	0.8	0.9	t	t	1.4
1032	1,8-cineole	t	0.1	0.1	0.4	t	t	0.2	-	t	t
1050	(E)-β-ocimene	0.9	0.3	0.2	0.5	t	0.1	0.1	1.1	0.7	0.1
1062	γ-terpinene	2.2	2.3	2.7	2.9	3.2	0.6	0.8	1.1	2.5	0.1
1068	cis-sabinene hydrate	2.3	1.6	2.1	3.2	2.3	0.5	1.1	0.7	1.4	0.5
1067	cis-linalool oxide(fur)	0.1	t	t	0.3	t	t	t	t	t	-
1088	terpinolene	1.1	1.2	1.3	1.2	1.2	0.3	0.6	0.8	1.0	0.7
1097	trans-sabinene hydrate	0.9	2.1	1.9	3.1	1.0	t	1.0	0.3	1.1	0.4
1098	linalool	1.0	t	t	3.1	1.0	0.4	1.0	1.5	0.3	0.2
1102	nonanal	-	-	t	-	-	-	-	t	-	t
1102	cis-thujone(α-thujone)	t	t	-	t	t	12.4	1.7	0.1	-	-
1114	trans-thujone(β-thujone)	0.4	t	t	0.2	0.1	6.7	12.7	0.7	0.1	-
1121	cis-p-menth-2-en-1-ol	0.5	0.4	0.4	0.5	0.5	0.2	0.2	0.2	0.6	0.2
1134	iso-3-thujanol	0.2	-	-	-	t	0.2	0.6	-	-	-
1140	trans-sabinol	-	-	-	0.5	0.3	4.3	5.0	0.7	0.3	-
1140	trans-p-menth-2-en-1-ol	-	0.2	0.4	-	-	-	-	-	-	0.2
1153	citronellal	0.1	t	t	-	-	-	-	0.2	0.4	-
1156	sabina ketone	0.1	t	t	0.2	0.1	t	t-	-	-	-
1166	coahuilensol	0.3	-	t	1.1	-	-	-	0.4	-	-
1177	terpinen-4-ol	5.0	3.9	4.4	5.9	4.8	1.6	2.1	1.4	7.2	2.9
1183	p-cymen-8-ol	t	-	t	0.1	t	t	t	-	-	-
1189	α-terpineol	0.2	0.2	0.2	0.5	0.2	t	0.1	0.1	0.3	0.2
1193	cis-piperitol	0.1	t	0.2	0.4	0.3	0.2	0.1	t	0.2	0.1
1205	trans-piperitol	0.2	0.2	0.2	0.3	0.3	t	0.2	-	0.2	0.1
1219	methyl coahuilensol	0.2	-	t	0.6	-	-	-	0.4	-	-
1228	citronellol	0.8	0.3	0.4	2.9	0.2	t	0.3	0.6	4.1	0.4
1257	linalyl acetate	t	0.1	0.1	0.8	0.8	t	0.6	0.2	-	0.3
1261	methyl citronellate	0.3	1.0	1.6	3.3	3.9	1.2	0.6	0.7	0.8	0.1
1285	bornyl acetate	t	t	t	t	t	t	-	t	-	0.4
1285	safrole	-	-	-	-	-	-	-	-	1.8	-
1287	trans-linalyl acetate	0.2	0.6	t	0.2	0.1	t	0.1	-	-	-
1290	trans-sabinyl acetate	1.7	0.5	0.3	0.4	0.4	39.8	39.6	35.0	t	-
1319	(E,E)-2,4-decadienal	t	t	t	0.3	t	0.2	t	-	-	-
1323	methyl geranate	0.4	t	0.3	0.4	0.4	0.9	t	0.3	0.1	0.1
1350	α-terpinyl acetate	0.1	t	t	0.2	0.2	t	0.1	0.1	-	0.2
1376	α-copaene	t	t	t	0.1	t	t	0.1	-	-	-
1401	methyl eugenol	-	13.2	t	0.3	5.8	4.1	0.4	-	1.1	-
1391	2E,4Z-me-decadienoate	t	0.4	0.5	t	0.8	0.3	t	-	-	-
1409	α-cedrene	-	-	-	-	-	-	-	-	-	0.2

	Table 1 (continued)	*J. sabina* var. *balkanensis*							*J. s.* var. *sabina*		
		low trans-sabinyl acetate (TSAC), and high sabinene					high TSAC, low sabinene		high TSAC	low TSAC	
RI	Compound	Tur-key	Greece		Bulg. east. Rhod	Bulg. Rila mts.	Rila Hi TSac	Rhod Hi TSac	SWZ Hi TSac	PYR Lo TSac	KAZ Lo TSac
1418	(E)-caryophyllene(β-caryophyllene)	t	t	0.1	t	0.2	t	0.1	-	-	-
1468	pinchotene acetate	t	-	-	0.5	-	-	-	0.1	-	t
1477	γ-muurolene	0.1	t	t	0.2	0.1	t	0.3	0.1	t	0.1
1480	germacrene D	0.1	t	t	t	t	t	0.3	-	0.1	-
1491	trans-murrola-4(14),5-diene	t	t	t	t	t	t	t	-	0.6	t
1493	epi-cubebol	0.1	t	t	0.2	0.2	t	0.3	0.1	0.3	-
1495	γ-amorphene	-	-	-	-	-	-	-	-	-	0.1
1499	α-muurolene	0.2	0.1	0.1	0.3	0.3	0.2	0.5	0.1	0.2	0.2
1513	γ-cadinene	0.4	0.5	0.4	0.8	0.6	0.4	1.7	0.3	0.8	0.3
1514	cubebol	t	t	t	t	t	t	t	-	-	-
1524	δ-cadinene	0.8	0.8	0.6	1.1	0.9	0.5	1.9	0.5	1.0	0.8
1538	α-cadinene	0.2		t	0.1	0.3	t	0.2	0.1	0.1	0.1
1549	elemol	0.6	t	t	t	1.9	0.5	0.1	0.8	2.1	0.1
1554	elemicin	0.1	6.0	t	t	2.3	6.4	0.2	-	0.4	-
1574	germacrene D-4-ol	2.6	1.4	1.8	2.2	2.4	0.5	2.5	1.4	0.7	1.1
1587	allo-cedrol	-	-	-	-	-	-	-	-	-	1.1
1596	cedrol	-	-	-	-	-	-	-	-	-	15.9
1606	β-oplopenone	0.7	0.7	0.3	0.6	1.1	0.5	0.4	0.1	0.3	-
1611	epi-cedrol	-	-	-	-	-	-	-	-	-	0.1
1627	1-epi-cubenol	0.1	t	t	t	0.2	0.1	0.2	t	0.4	-
1632	α-acorenol	-	-	-	-	-	-	-	-	-	0.2
1640	epi-α-cadinol	0.4	0.3	0.3	0.5	0.5	0.3	0.8	0.2	0.3	0.3
1640	epi-α-muurolol	0.4	0.4	0.3	0.5	0.6	0.3	0.8	0.2	0.2	0.3
1645	α-muurolol	0.1	0.1	t	0.2	0.2	0.1	0.3	0.1	0.1	0.1
1649	β-eudesmol	t	-	-	-	0.6	0.2	-	-	0.2	-
1652	α-eudesmol	t	-	-	-	0.6	t	-	-	0.3	-
1653	α-cadinol	1.0	0.8	0.7	1.3	1.2	0.9	1.8	0.6	0.5	0.9
1689	shyobunol	0.4	t	0.3	t	0.5	0.1	0.2	0.1	t	0.1
2054	abietatriene	t	t	t	t	t	t	t	t	t	t
2080	abietadiene	0.2	0.2	0.4	0.3	t	t	0.2	t	0.1	t
2288	4-epi-abietal	0.4	0.3	0.5	0.7	0.7	0.3	0.5	t	0.1	t
2302	abieta-7,13-dien-3-one	1.0	0.7	1.3	1.9	2.0	0.5	0.7	0.1	0.1	0.2
2325	trans-ferruginol	t	t	t	t	0.2	t	t	t	t	t
2343	4-epi-abietol	t	t	t	0.3	0.1	t	t	t	t	t

RI = Kovat's Retention Index on DB-5(=SE54) column using alkanes. Compositional values less than 0.1% are denoted as traces (t). Unidentified components less than 0.5% are not reported.

Table 2. Comparisons of the per cent total oil for leaf essential oils for *J. sabina* var. *balkanensis* from central Italy. Components that separate the samples are highlighted. *J. s.* var. *sabina* as per Table 1.

		J. sabina var. *balkanensis*, central Italy						*J. sabina* var. *sabina*		
		low trans-sabinyl acetate			high trans-sabinyl acetate			HiTSac	LoTSac	
RI	Compound	15503	15501	15500	15505	15504	15502	SWZ	PYR	KAZ
931	α -thujene	0.9	0.4	0.9	0.4	0.3	0.4	0.9	1.0	0.6
939	α-pinene	1.6	1.4	3.5	2.4	1.8	1.4	2.0	1.8	15.8
953	camphene	t	t	t	t	t	t	t	t	0.3
976	sabinene	35.7	28.4	40.2	13.9	9.6	14.8	34.8	54.9	42.6
980	β-pinene	0.1	0.2	t	0.2	0.2	0.2	t	t	0.7
991	myrcene	1.2	2.2	2.7	2.0	1.8	2.4	4.2	3.1	3.8
1005	α-phellandrene	t	t	t	t	t	t	t	0.1	t
1011	δ-3-carene	t	t	t	t	t	t	-	0.1	0.2
1018	α-terpinene	1.2	0.8	1.0	0.7	0.4	0.5	1.0	1.5	0.7
1026	p-cymene	0.3	0.2	0.2	0.2	0.2	0.2	0.2	0.4	0.1
1031	limonene	0.9	0.8	0.9	0.6	0.6	0.7	3.0	2.4	2.1
1031	β-phellandrene	0.8	0.7	0.9	0.6	0.5	0.6	t	t	1.4
1032	1,8-cineole	0.1	0.1	t	0.2	t	t	-	t	t
1050	(E)-β-ocimene	t	t	t	0.1	t	0.2	1.1	0.7	0.1
1062	γ-terpinene	2.0	1.6	1.7	1.1	0.7	0.8	1.1	2.5	0.1
1068	cis-sabinene hydrate	1.6	2.0	1.4	0.7	0.7	0.9	0.7	1.4	0.5
1067	cis-linalool oxide (furan)	-	-	-	-	-	-	t	t	-
1088	terpinolene	0.8	0.7	0.8	0.6	0.5	0.6	0.8	1.0	1.0
1097	trans-sabinene hydrate	2.0	1.5	1.0	0.6	0.7	0.6	0.3	1.1	0.4
1098	linalool	t	t	t	0.3	0.2	t	1.5	0.3	0.2
1102	cis-thujone(α-thujone)	t	t	t	3.5	3.3	8.0	0.1	-	-
1106	cis-rose oxide	t	t	-	t	-	t	-	-	-
1114	trans-thujone(β-thujone)	0.2	0.9	1.1	8.4	3.0	2.2	0.7	0.1	-
1121	cis-p-menth-2-en-1-ol	0.4	0.4	0.3	0.2	0.2	0.2	0.2	0.6	0.2
1122	trans-rose oxide	t	-	-	-	-	-	-	-	-
1134	iso-3-thujanol	t	t	t	0.1	t	0.2	-	-	-
1140	trans-sabinol	0.1	0.4	0.3	1.0	1.7	1.8	0.7	0.3	-
1140	trans-p-menth-2-en-1-ol	0.1	-	-	-	-	-	-	-	0.2
1153	citronellal	0.9	t	t	-	-	-	0.2	0.4	-
1156	sabina ketone	0.1	0.6	0.3	0.1	t	t	-	-	-
1166	coahuilensol	t	0.3	0.2	t	t	t	0.4	-	-
1177	terpinen-4-ol	4.3	3.9	2.9	2.0	1.7	1.7	1.4	7.2	2.9
1183	p-cymen-8-ol	t	t	t	t	t	t	-	-	-
1189	α-terpineol	0.2	0.2	0.1	t	1.7	0.1	0.1	0.3	0.2
1193	cis-piperitol	t	0.3	0.2	0.1	0.3	0.2	t	0.2	0.1
1205	trans-piperitol	0.1	0.2	t	t	t	t	-	0.2	0.1
1219	methyl coahuilensol	0.3	0.3	0.2	-	t	0.1	0.4	-	-
1228	citronellol	7.5	6.9	3.5	2.6	2.5	t	0.6	4.1	0.4
1253	trans-sabinene hydrate acetate	0.1	-	-	-	-	-	-	-	-
1257	linalyl acetate	t	0.2	t	t	t	t	0.2	-	0.3
1261	methyl citronellate	2.1	8.8	5.9	5.1	6.6	0.3	0.7	0.8	0.1
1285	bornyl acetate	0.6	0.2	0.2	t	t	t	t	-	0.4
1285	safrole	-	-	-	-	-	-	-	1.8	-
1290	trans-sabinyl acetate	0.3	6.8	7.3	36.0	47.4	36.8	35.0	t	-
1319	(2E,4E)-decadienal	0.1	t	t	t	t	0.1	-	-	-
1323	methyl geranate	t	0.3	0.3	0.8	1.0	t	0.3	0.1	0.1
1350	α-terpinyl acetate	t	t	t	t	t	t	0.1	-	0.2
1374	isoledene	0.1	t	t	t	t	t	-	-	-
1376	α-copaene	t	t	t	t	t	0.1	-	-	-
1391	(2E,4Z)-me-decadienoate	0.1	1.0	0.7	0.5	0.6	t	-	-	-

		J. sabina var. *balkanensis*, central Italy						*J. sabina* var. *sabina*		
		low trans-sabinyl acetate			high trans-sabinyl acetate			HiTSac	LoTSac	
RI	Compound	15503	15501	15500	15505	15504	15502	SWZ	PYR	KAZ
1401	methyl eugenol	5.3	t	t	t	0.1	11.2	-	1.1	-
1409	α-cedrene							-	-	0.2
1418	(E)-caryophyllene(β-caryophyllene)	0.1	0.3	0.2	t	0.1	0.1	-	-	-
1451	trans-murrola-3,5-diene	0.6	0.4	0.3	t	t	0.2	-	-	-
1468	pinchotene acetate	0.2	-	-	t	-	0.2	0.1	-	t
1475	trans-cadina-1(6),4-diene	0.6	0.3	0.3	0.2	t	t	-	-	-
1477	γ-muurolene	t	t	t	t	t	t	t	t	0.1
1480	germacrene D	-	-	-	t	0.4	-	-	0.1	-
1491	trans-murrola-4(14),5-diene	1.7	1.2	0.9	t	0.1	0.6	-	0.6	t
1493	epi-cubebol	0.9	0.5	0.5	t	t	0.3	0.1	0.3	-
1499	α-muurolene	0.2	0.3	0.2	t	0.2	0.2	-	0.2	0.2
1513	γ-cadinene	2.0	1.5	1.3	0.2	0.3	0.6	0.3	0.8	0.3
1514	cubebol	1.6	1.5	1.2	0.2	0.2	0.5	-	-	-
1524	δ-cadinene	1.2	1.2	1.0	0.7	0.7	0.7	0.5	1.0	0.8
1528	zonarene	0.3	0.2	0.2	t	t	0.1	-	-	-
1538	α-cadinene	t	t	t	t	t	t	0.1	0.1	0.1
1549	elemol	t	t	t	t	t	t	0.8	2.1	0.1
1554	elemicin	5.4	-	-	0.2	0.2	1.0	-	0.4	-
1574	germacrene D-4-ol	0.9	4.1	3.2	1.5	1.8	1.2	1.4	0.7	1.1
1587	trans-murrol-5-en-4- α-ol	1.1	t	t	t	-	0.3	-	-	-
1587	allo-cedrol	-	-	-	-	-	-	-	-	1.1
1596	cedrol	-	-	-	-	-	-	-	-	15.9
1606	β-oplopenone	0.7	0.8	0.7	0.3	0.4	0.4	0.1	0.3	-
1611	epi-cedrol	-	-	-	-	-	-	-	-	0.1
1627	1-epi-cubenol	1.6	0.8	0.8	0.1	0.1	0.5	t	0.4	-
1632	α-acorenol	-	-	-	-	-	-	-	-	0.2
1640	epi-α-cadinol	0.3	0.6	0.5	0.3	0.4	0.2	0.2	0.3	0.3
1640	epi-α-muurolol	0.3	0.5	0.5	0.4	0.4	0.2	0.2	0.2	0.2
1645	α-muurolol	t	0.1	0.1	t	t	t	0.1	0.1	0.1
1649	β-eudesmol	-	-	-	-	-	-	-	0.2	-
1652	α-eudesmol	-	-	-	-	-	-	-	0.3	-
1653	α-cadinol	0.5	1.2	1.0	1.1	1.3	0.5	0.6	0.5	0.9
1689	shyobunol	t	t	t	0.6	0.8	0.3	0.1	t	0.1
2054	abietatriene	0.1	0.3	t	t	t	t	t	t	t
2080	abietadiene	0.1	0.2	t	t	t	t	t	0.1	t
2288	4-epi-abietal	0.3	1.2	0.8	0.5	0.4	0.2	t	0.1	t
2302	abieta-7,13-dien-3-one	0.4	2.4	1.6	0.5	0.5	0.6	0.1	0.1	0.2
2325	trans-ferruginol	t	t	t	t	t	t	t	t	t
2343	4-epi-abietol	t	0.1	t	t	t	t	t	t	t

RI = Kovat's Retention Index on DB-5(=SE54) column using alkanes. Compositional values less than 0.1% are denoted as traces (t). Unidentified components less than 0.5% are not reported.

Correcting a Previously Published Error in Soil Salinity Comparisons Reported from a West Texas Salt Marsh

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ABSTRACT

In 2007, a book chapter was published concerning monthly changes in soil salinity levels over an annual plant growth cycle in an inland salt marsh. Two figures were published to compare surface plots showing soil salinity in the salt marsh. Unfortunately, one figure was duplicated, so rather than showing a comparison of two techniques, only one was shown. This article is to correct that error. Spatial and seasonal fluctuations of the soil salinity levels in an inland salt marsh were measured and then examined with geographic information system software to gain insight into the cause of the distribution of the plant communities in the marsh. Surface plots were interpolated using the inverse distance weighted method (ArcView 3.3) and the ordinary kriging method (ArcGIS 8). Using the same data, ordinary kriging generated a gradual, smooth surface, which was not correctly published, while the inverse distance weighted surface was irregular. Using the ordinary kriging method, the mean error and root-mean-square error statistics were closer to zero indicating a better estimation of the soil salinity. Generated surfaces showed seasonal fluctuation and well defined spatial changes. Lowest elevation in the center of the salt marsh had lowest levels of soil salinity, while the marsh edges at higher elevations had increased soil salinity. Spatial patterns of soil salinity seem to depend on seasonality of rainfall, plant activity and soil water content. Local surface anomalies often mask these patterns. Use of ordinary kriging and interpolation reduced some of the masking effects and better revealed salinity patterns. Unfortunately, this was stated in the published chapter, but the supporting figures were not presented correctly. Published on-line www.phytologia.org *Phytologia 101(1): 46-57 (March 21, 2019)*. ISSN 030319430.

KEY WORDS: soil salinity, geographic information systems, interpolation, kriging, IDW, arid environments, inland salt marshes, spatial changes.

Because our intent is to correct an error in a published document, we will present a limited amount of material about the west Texas salt marsh, the descriptive characteristics of the marsh and techniques used. Readers should refer to the original publication and some others that concern this particular marsh and it characteristics (Grunstra and Van Auken 2007).

Geographical Information Systems (GIS) and Global Positioning Systems (GPS) provide new ways to investigate and display areas both spatially and temporally. Surface contour plots are tools readily available with the emergence of cheaper GIS software. A surface contour plot allows a limited point data set to be expanded to display estimated values at any point within a study area. There are numerous interpolation methods available for the creation of these surface contours (Lam 1983; Isaaks and Srivastava 1989; Burrough and McDonnell 1998). Each interpolation method allows the user to modify characteristics and reduce variation in the output surface. This paper compared output surfaces created using two common

interpolation techniques, including the Inverse Distance Weighted method (IDW) and Ordinary Kriging (Franzen and Peck 1995; Weisz et al. 1995). The paper demonstrates how these surfaces were applied to field research by examining the spatial and temporal fluctuations of soil salinity levels in an inland salt marsh. More specifics are presented in the previously published paper (Grunstra and Van Auken 2007).

The IDW method produces accurate surface interpolation as long as a regular distribution is used (ESRI 2000; Johnston et al. 2001; Mitchell 2009). Uneven distributions may produce sharp peaks or troughs in the output surface (ESRI 2000; Johnston et al. 2001; Mitchell 2009). The ArcGIS 8 software allows the use of geostatistics to create surface contour plots. Kriging is a geostatistical technique that can explain the variation of a surface (Isaaks and Srivastava 1989; Burrough and McDonnell 1998; ESRI 2000; Johnston et al. 2001; ESRI 2003; Mitchell 2009). Output from this model includes a mean error, root-mean-square error, average standard error, and the root mean square standardized error (Johnston et al. 2001). Many studies have compared the performance of these methods suggesting careful consideration when determining the method and settings to be used with a given data set.

Many studies of salt marshes have incorporated GIS into their analysis, but none have considered this west Texas salt marsh. The Diamond Y Spring is located on a 6.1 km^2 nature preserve owned by the Nature Conservancy of Texas, approximately 16 km north of Fort Stockton, Texas (Figure 1). The Diamond Y Spring is the last major spring still flowing in Pecos County, Texas (Veni 1991). The Preserve protects six federally endangered or threatened species including the Puzzle Sunflower (*Helianthus paradoxus)*, two fish and three snails (McDonald 1999; TPWD 2003; Bush and Van Auken 2004). The types of soil, water chemistry, as well as the plant communities are all indicators that a large part of the Diamond Y Spring Preserve is a salt marsh.

The Preserve is semi-arid karst with an average precipitation of 33 cm/year and an evaporative rate of 204 cm/year (Larkin and Bomar 1983), with three saline aquifers underlying the area (Figure 1) (Veni 1991; Small and Ozuna 1993; Boghici 1997; USGS 2002). The surface flow is east toward the Pecos River and ephemeral due to low rainfall and high evaporation (Van Auken 1998; Grunstra 2002; Hart 2002). Plant communities within the salt marsh seem to be in fairly distinct locations due to species specific water requirements, salt tolerance, or ability to out-compete rivals (Chapman 1974; Niering and Warren 1980; Bertness 1991; Van Auken and Bush 1998; Bush 2002; Grunstra 2002).

PURPOSE

To gain a greater understanding of the distribution of the salt marsh vegetation, soil salinity data were gathered. Two different geographic information system software interpolation methods were compared. The surface contour plots interpolated values to fill in the entire study area and provide a representative overview of the soil salinity levels throughout the salt marsh.

METHODS

Two different geographic information system software interpolation methods were used on the same input variables to generate two visually different surface contour plots to examine the most useful interpolated surface from the data. These methods will be abbreviated and are mainly presented so the reader has some understanding how the comparative figures were established. More detail is presented in the original publication (Grunstra and Van Auken 2007). A GeoExplorer III GPS receiver was used to map the study area including 7 transects with 87 observation points (Figures 2 and 3). The GPS field data were used to create point shapefiles in the ArcView 3.3 and ArcGIS 8 software (ESRI 1999; Ormsby and Alvi 1999; Mitchell 2009; Allen 2011).

Soil samples from each site were collected monthly beginning in January 2002 and continuing until October 2002 for 870 total samples. Approximately 300 g of surface soil was collected from the top 1 cm of soil below surface litter (TAES 1983). When under water, a sample of water was collected. The soil was collected approximately 2 m from each observation point in a cross pattern. Samples were placed in plastic bags and sealed to prevent evaporation then taken to the laboratory for analyses. Soil salinity was measured by making a 1:1 paste (soil:de-ionized water, V:V) and measured with a salinity probe (TAES 1983; Westerman 1990; Rowell 1994;). These measurements were entered into the attribute table of the GIS shapefiles created in ArcView 3.3 and ArcGIS 8 from the collected GPS field sampling site locations.

Soil salinity point measurements were converted to a raster grid to produce a continuous surface or contour plot across the study area. In ArcView 3.3, the Spatial Analyst extension was used to do this interpolation by the Inverse Distance Weighted (IDW) method. In ArcGIS 8, the Geostatistical Analyst extension was used to create the surface plots by the kriging method. Several iterations were performed for each interpolation method using various initial settings of neighborhood size of analysis, lag spacing, and power of magnification. This was performed in a systematic fashion in order to produce the most logical and representative interpolated surface for each method.

The settings used for the IDW surface displayed in this study were a neighborhood size of 15 with a power magnification of 3. The settings that produced the best cross-validation error statistics for the ordinary kriging method were for an elliptical search neighborhood with no offset and divided into 4 sectors including 5 points per sector. Both methods allowed for the display and investigation of spatial and temporal trends and patterns of soil salinity levels in the marsh. The outputs from the two separate interpolation methods were compared visually as well as by the error statistics produced through cross-validation. The ArcGIS 8.0 software was used to perform cross-validation and generate the mean prediction error and the root-mean-square prediction error.

RESULTS

The ArcView 3.3 soil surface salinity contour plots created using the IDW method showed a range of salinity levels from a low of 3 ppt to a high of 43 ppt (Figure 2). The solid black areas represent the highest salinity levels while lower levels are shown with various patterns. High salinity levels were found along the borders or edges of the study area especially in the northwest with lower salinity levels found towards the center or lower elevations of the marsh associated with the drainage (Figure 2). The surface soils in the eastern half of the study area consistently showed large areas with soil salinity levels in the 3 - 10 ppt salinity range. There was little variation in the soil salinity levels in these locations through the ten-month study period.

Surface soil salinity levels greater than 20 ppt were mainly along the edge of the northwestern part of the study area (Figure 2). Using the IDW method seemed to put more weight on the central measurements on a given transect, thus if the central measurement was very low, then that point showed up lighter in the figures with associated values used to extrapolate the central measurement which were darker indicating higher extrapolated salt values. Accordingly, there were a larger number of higher or lower isolated points or associated map values and the map using the IDW procedure did not show more general and uniform gradients (Figure 2). There seemed to be higher variance in the map when the IDW procedure was used.

The surface contours created using the ordinary kriging method were smoother, showed less variation or were not as ragged as those created using the IDW procedure (comparing Figures 2 and 3; note, comparisons were not possible in the earlier publication because the equivalent of Figure 3 was not correctly presented). In addition, they also show a large portion of the study area with soil salinity in the range from 5 - 10 ppt (Figure 3). The majority of the higher levels of soil salinity in the range of 25 - 43 ppt were found along the northwestern border of the study area with occasional occurrences near the southwestern

border. February and October show most of the study area with lower levels of salinity with very little area in the higher ranges (Figure 3). April and September show larger areas with higher levels of salinity covering more of the study area than the rest of the time period (Figure 3). The trend shows higher levels always near the borders and lower levels towards the middle of the study area.

Through visual examination both general differences and similarities of the surface plots created using the IDW and ordinary kriging methods could be observed (Comparing Figures 2 and 3, see previous note). In the IDW surface plots, the sampling points and transects can more readily be observed (Figure 2). The ordinary kriging method depicts more gradual and smoother transitions between soil salinity values (Figure 3). The IDW procedure depicts a larger surface area of the salt marsh covered by the lower values of 3 - 5 ppt while the ordinary kriging method estimates those same areas to have slightly higher values between 5 - 10 ppt (Figures 2 and 3). On the northern border, the IDW method shows localized concentrations of high soil salinity around the sampling locations while the ordinary kriging method shows a banding pattern in those same areas. The IDW method tends to show less area covered by higher salinity and more area covered with lower salinity while the ordinary kriging method tends to show the reverse. Although both methods of visual representation show similar trends, the ordinary kriging surface contour plots are much smoother (Figure 3, not seen in previous publication because of figure duplication). The two methods were evaluated by comparing the overall mean prediction error and root-mean-square prediction error for their surface contour plots (Table 1). The ordinary kriging method consistently produced values closer to zero indicating it would generally yield a better estimation of the soil salinity.

DISCUSSION

The surface contour plots available in GIS software provide many new ways to investigate and display diverse results. They facilitate the ability to produce surface contour plots which can estimate and display values across a large surface area from a limited point data set (Isaaks and Srivastava 1989; Burrough and McDonnell 1998). This allows investigators to quickly identify spatial and temporal patterns and trends as well as possible interactions and influences that different factors may have on plants in their study area which can then be examined more critically.

The GIS user has numerous interpolation techniques from which to choose depending on the software package selected (Isaaks and Srivastava 1989; Burrough and McDonnell 1998; Johnston et al. 2001; Mitchell 2009). Furthermore, variables such as the neighborhood size of analysis, lag spacing and other settings can be changed within a given interpolation method. The surface contour plots created by these methods may show similar characteristics and trends but will often produce visually dissimilar surface contours with considerable differences at specific locations (Brodsky et al. 2001; Bucher and Vckovski 1995; Gotway et al. 1996; Dille et al. 2003; Jones et al. 2003; Kravchenko 2003; Mueller et al. 2004). These dissimilarities are inherent to the mathematical procedures used to create the final surface contour such as the mathematical equations, calculations and estimations used by the method (Isaaks and Srivastava 1989).

In this study, both the IDW and the ordinary kriging methods were used to provide examples of different visual outputs created using the same data. The kriging method produced a smooth, more regular interpolated surface, whereas the IDW method produced a surface that was more strongly influenced by local measurements or the values of the specific soil samples (Comparing Figures 2 and 3). Most likely this was caused by the irregularly spaced pattern of the observation sites which were considerably closer in the north-south direction than the west-east direction. The IDW method produces a fairly exact surface interpolation as long as a regularly distributed sampling pattern is employed (Ormsby and Alvi 1999; ESRI 2000; Johnston et al. 2001; Mitchell 2009; Allen 2011). High point value variance or uneven distribution in the sampling patterns often produces sharp peaks or troughs in the output surface (Ormsby and Alvi 1999; ESRI 2000; Johnston et al. 2001; Mitchell 2009). Care must be taken with different methods and different settings when creating an interpolated surface in order to avoid interjecting various biases (Gotway

et al. 1996; Dille et al. 2003; Jones et al. 2003; Kravchenko 2003; Mueller et al. 2004). The results can be useful both visually and in predicting values for variables (in this case salinity) between sample points.

When deciding upon the appropriate interpolation method to use to investigate or display data, one must critically evaluate various methods and program settings in order to obtain the best visual representation of logical values between the sample points (Bucher and Vckovski 1995; Gotway et al. 1996; Brodsky et al. 2001; Dille et al. 2003; Jones et al. 2003; Kravchenko 2003; Mueller et al. 2004). In the current study, the IDW results are ragged with little smoothing between sampling locations because the IDW method uses exact interpolation with contours formed on the specific measurements entered into the program. Consequently, when the plots are examined it is easy to see where the measurements were actually made. For example, when one examines Figure 2, the locations of the transects (and many soil collection sites) are obvious because of the sharp local differences in the places on the plots and the consequent lack of smoothing.

Using the kriging method, the northern and southern borders of the salt marsh consistently show the highest levels of soil salinity. These same areas coincide with elevations that are higher than the center of the salt marsh. The higher salinity values at the northern and southern borders of the salt marsh are attributed to a shallow soil or deeper water table that allows the soil to dry and therefore increase the soil surface salt concentration. More of the salts would be washed out of the surface soils in an area with the water table closer to the surface (Neill 1993; Ala et al. 1995). The areas with high soil salinity can be seen to grow larger as the water table gets deeper and dry areas of the marsh increase in size (see Grunstra 2002). The same high surface soil salinity areas then recede when the water table rises and the salts are flushed out of the soil by the surface water.

Surface soil salinity in the Diamond Y Spring salt marsh was previously found to be at its lowest level in early spring and increased during the summer months (Schmidt 1986; Van Auken and Bush 1993, 1995, 2006). This fluctuation in surface soil salinity was thought to occur in unison with the cyclic pattern of the water table. A higher water table would allow for more of the salts to be washed out of the surface soils while a lower water table would allow the soil to dry and therefore increase the surface soil salt concentration. Soil salinity reduced production of a cool season grass until spring flooding decreased soil salinity (Neill 1993). High levels of soil water allowed salts to be distributed throughout the soil profile while low levels soil water caused salt accumulation in the upper soil layers due to the high evaporation rates (Ala et al. 1995).

The expected annual cycle of salinity in the Diamond Y Spring salt marsh was not as noticeable as expected due to variation in the annual rainfall pattern (NCDC 2002). January of the study year received no precipitation when it usually receives approximately 2 cm while June and July received greater amounts of precipitation than normal. The month of August showed very little precipitation (0.4 cm) compared to the mean precipitation expected during that month (5 cm). The monthly mean soil salinity fluctuations were observed but they were not as large as expected (Figures 2 and 3).

Temporal and spatial distributions of soil moisture, pH, and ionic composition were significant in determining plant community locations in a Mediterranean salt marsh (Rogel et al. 2001). Surface salinity had a negative effect on all growth parameters and aboveground dry mass of *Helianthus paradoxus* at the Diamond Y Spring Preserve (Bush 2002, 2005). In addition these effects were time dependent. Spatial and temporal fluctuations in three halophyte species in upper coastal salt marsh communities were influenced by saline stress and soil nutrient level (Omer 2004). Temporal change in soil salt levels were found to determine plant community locations along the shoreline of a desert basin lake (Toft and Elliot-Fisk 2002). Soil salinity and moisture were also found to effect the spatial and temporal variation in plant germination and establishment in upper tidal marshes of three southern California wetlands (Noe and Zedler 2001). Plant zonation was related to spatial and temporal variations in soil salinity in southeastern Spain

(Ortiz et al. 1995). Vegetation distribution was also determined by soil salinity in spring fed salt marshes in western Utah (Bolen 1964) and around the Great Salt Lake (Flowers 1934).

Through the use and application of GIS, greater knowledge of the spatial and seasonal fluctuations of the soil salinity levels in salt marshes has been obtained. GIS interpolations have been used to determine the spatial dynamics of soil salinity in arid and semiarid regions (Jordan et al. 2004; Shi et al. 2005) as well as to determine the temporal and spatial variability of soil salinity in coastal saline fields and in cotton fields irrigated with low-quality water (Cetin and Kirda 2003). In the current study, the surface contours created for the Diamond Y Spring salt marsh have shown seasonal fluctuations and spatial distribution in the soil salinity across the salt marsh. The varying soil salinity levels probably indicate zonation and probable locations of salt marsh vegetation although this inland salt marsh and is most likely coupled with the interaction of water level at different points in time during the growing season.

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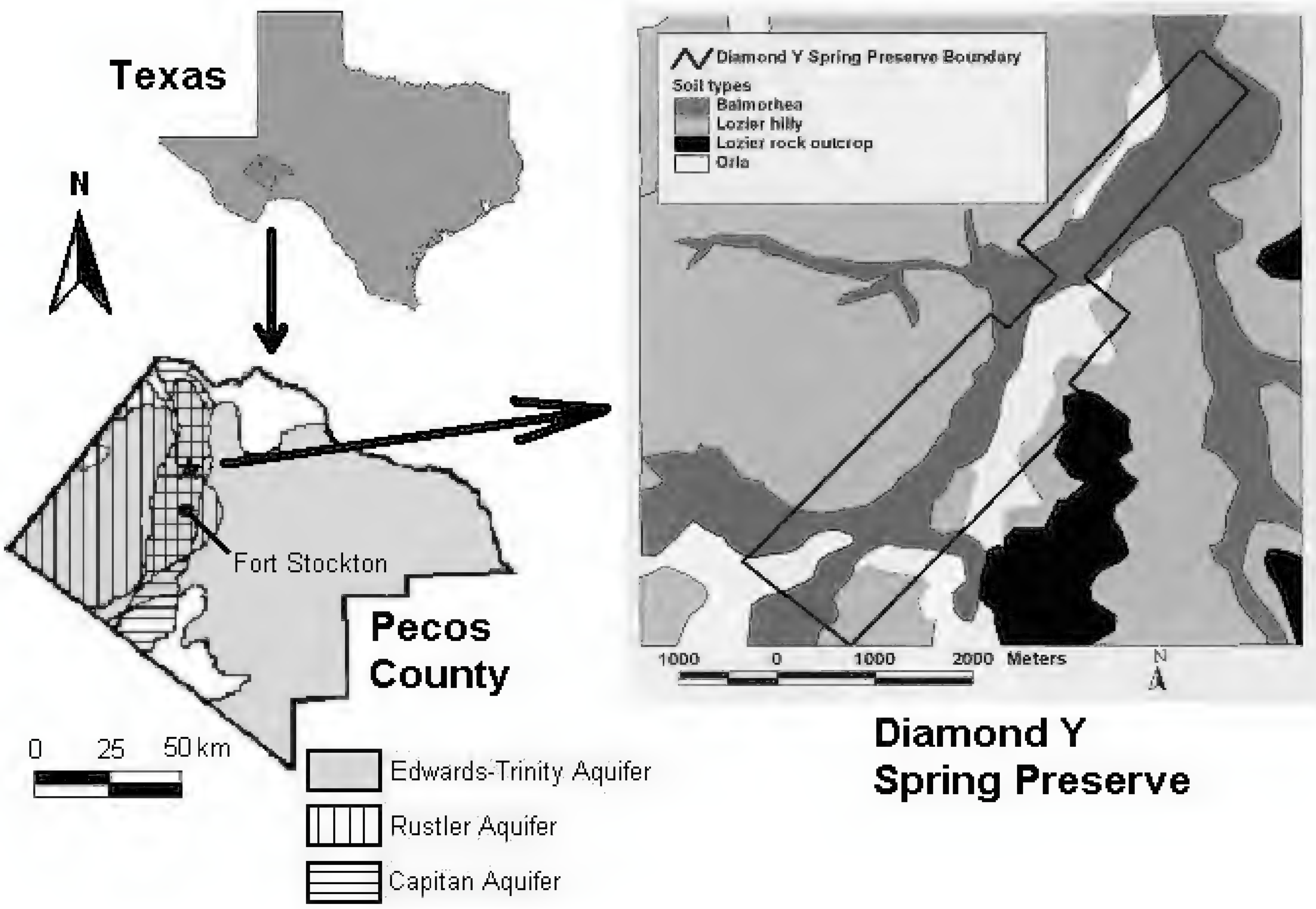

Figure 1. Pecos County is located in west Texas. The major aquifers underlying the Fort Stockton area and Pecos County, Texas include the Edwards-Trinity, Rustler and Capitan Reef. Soil types present in the area of the Diamond Y Spring Preserve include Balmorhea, Orla, and Lozier association soils (Rives 1980).

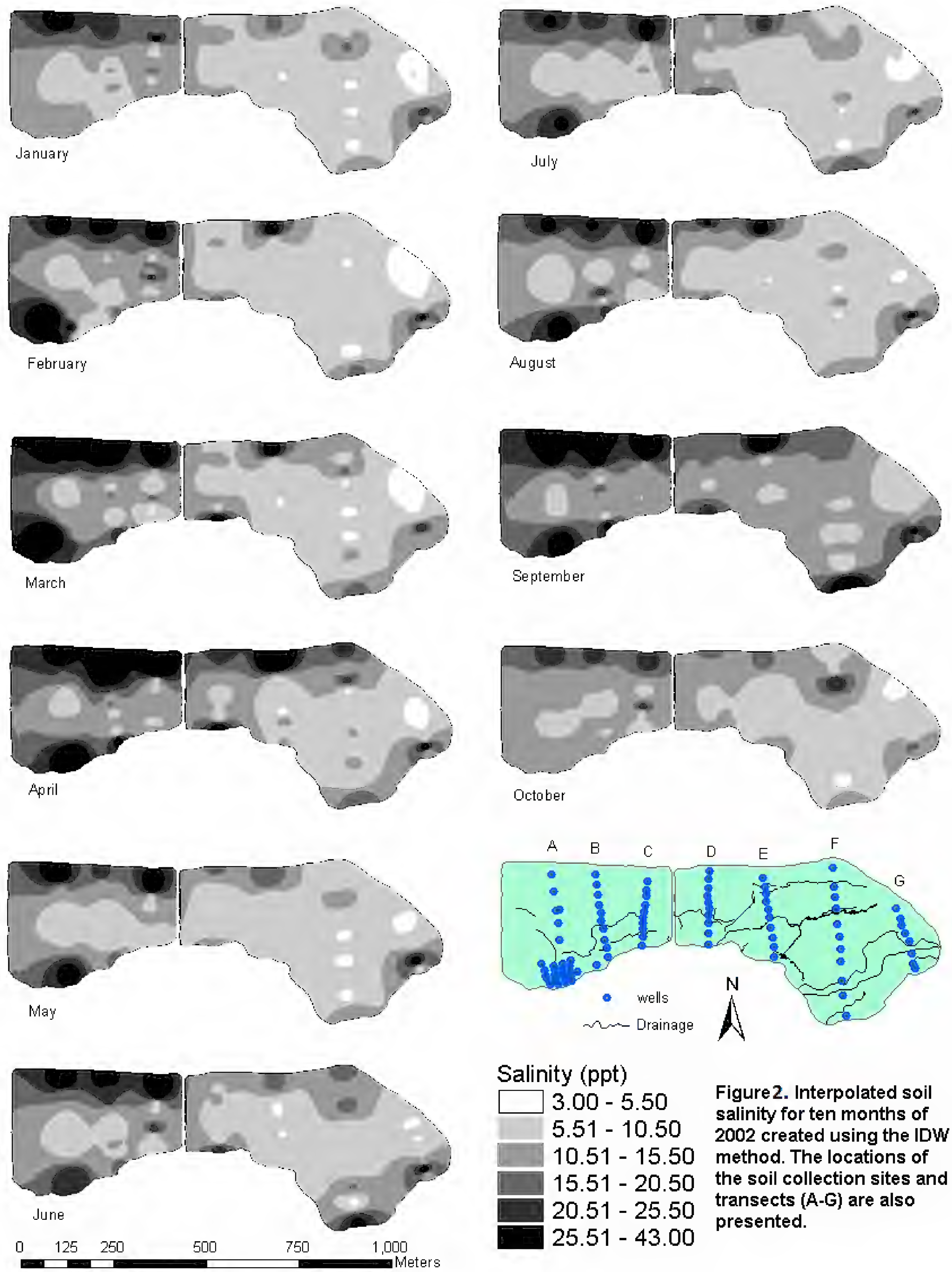

Figure2. Interpolated soil salinity for ten months of 2002 created using the IDW method. The locations of the soil collection sites and transects (A-G) are also presented.

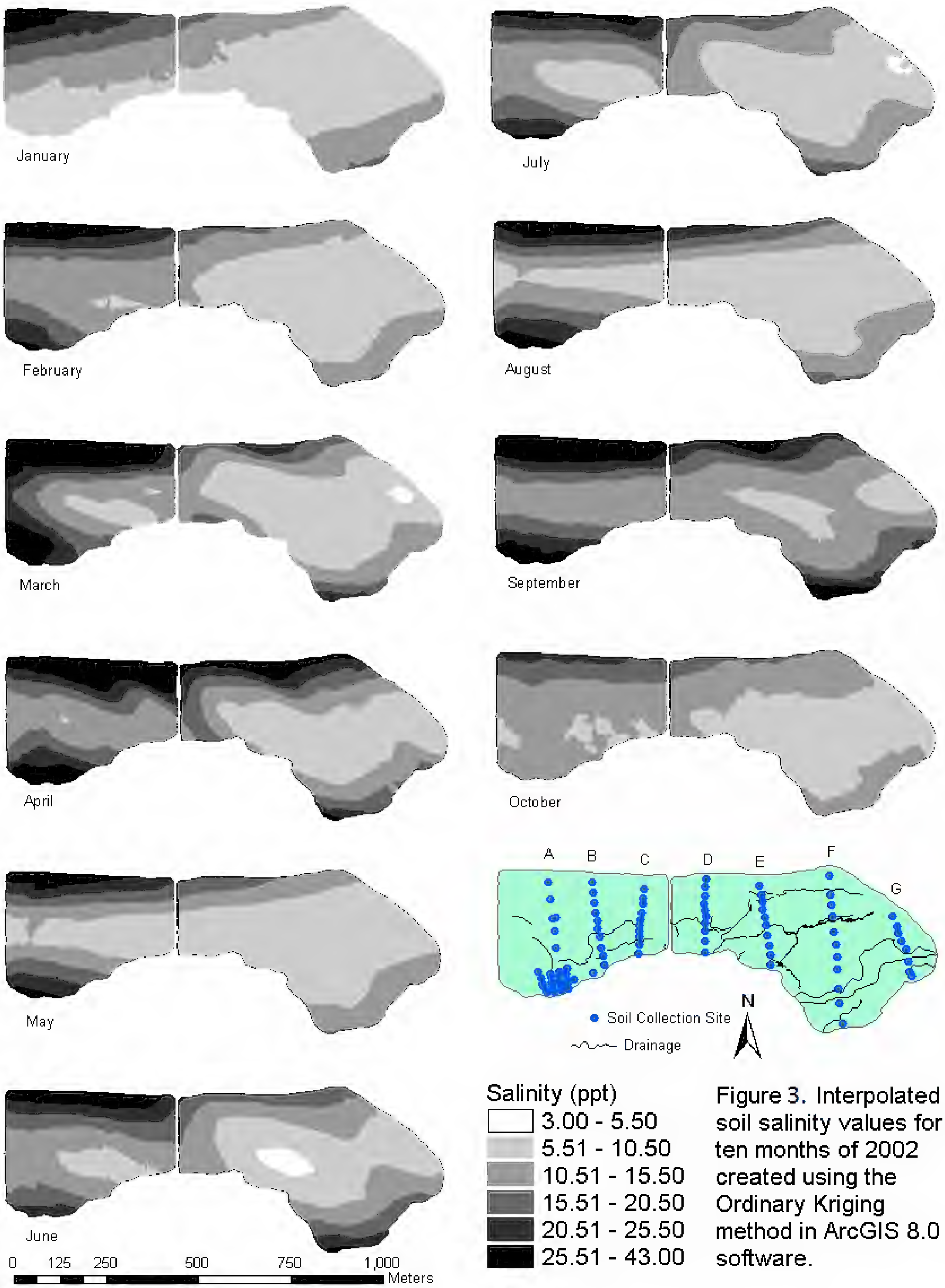

Figure 3. Interpolated soil salinity values for ten months of 2002 created using the Ordinary Kriging method in ArcGIS 8.0 software.

Month	Mean		Root-Mean-Square	
	IDW	Kriging	IDW	Kriging
January	-0.15	0.05	5.48	5.10
February	-0.69	-0.05	7.31	6.97
March	-1.13	-0.06	7.67	7.30
April	-1.26	-0.29	10.1	8.57
May	-1.02	-0.13	7.63	6.38
June	-1.06	-0.18	8.27	6.59
July	-0.89	-0.36	6.17	5.20
August	-0.87	0.01	7.74	6.07
September	-1.33	-0.27	7.65	6.76
October	-0.46	-0.05	5.97	4.97

Table 1. Mean and Root-Mean-Square error statistics generated for the interpolated surfaces created using the IDW and ordinary kriging methods. The ordinary kriging method consistently produced values closer to zero indicating it would generally yield a better estimation of the soil salinity.

Inheritance of single copy nuclear genes (SCNGs) in artificial hybrids of *Hesperocyparis arizonica* x *H. macrocarpa*: Potential for utilization in the detection of hybridization in natural populations

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ABSTRACT

Analyses were performed on 18 artificial hybrids from a cross of *Hesperocyparis arizonica* (male parent) *x H. macrocarpa* (female parent) using 9 single copy nuclear genes (SCNGs). Three SCNG were found to be informative: myb, 4CL and CnAIB2. Gene myb contained 5 variable sites, of which site 89 was homozygous (CC, TT) as was site 261 (GG, AA) and useful for the detection of hybridization. All 18 hybrids were heterozygous (CT and GA) at these 2 sites as predicted in hybrids. 4CL contained 8 variable sites, of which 1 site (591) was homozygous (TT, CC) and all 18 hybrids were heterozygous (TC) at this site as expected. CnAIP2 had two variable sites: 301 (AA, AC) and 554 (AG, AA). For site 301, 8 hybrids were AA, and 10 were AC as expected. For site 554, 10 hybrids were AA and 8 were AG, so neither would be useful for unequivocally identifying hybrids. The inheritance of variable sites for the three SCNGs followed simple co-occurrence. Examination of myb in the 18 hybrids revealed 2 cases of cross-over in the pollen gametes. Published on-line www.phytologia.org *Phytologia 101(1):58-66 (March 21, 2019)*. ISSN 030319430.

KEY WORDS: *Hesperocyparis arizonica, H. macrocarpa,* Cupressaceae, hybrids, single copy nuclear genes, SCNG, inheritance.

Recently, Adams, Miller and Low (2016) analyzed the inheritance of nrDNA in artificial hybrids between *Hesperocyparis arizonica* and *H. macrocarpa* from New Zealand. Sequencing nrDNA of parents (*Hesperocyparis arizonica, H. macrocarpa)* found their nrDNA differed at 8 sites. Analysis of 18 artificial hybrids, revealed each of the hybrids had nrDNA that was heterozygous at each of the 8 sites. However, the peak ratios in the chromatograms were not 1:1 as expected, but varied from 1:1 to 3:1, usually being more like *H. arizonica*. Principle Coordinates Ordination (PCO) of the variation in the peak heights revealed four groups of hybrids that seemed to be associated with chromosome inheritance. However, PCO clearly distinguished the parents and the hybrids. But, the ordination of hybrids closer to *H. arizonica*, could lead one to interpret that some introgression was occurring, when, in fact, there were only hybrids in the PCO.

However, nrDNA spacer regions have been reported to exhibit some oddities in inheritance. The conserved nature of the multi-copy nrDNA (thousands of copies per cell) seemed to be due to concerted

evolution (Liao, 1999). Liao (1999) argues that because rRNAs are structural molecules, multiple gene copies are necessary to supply the demand for ribosomal subunits in the cell. Because these sub-units function only when assembled into a large complex, homogeneity of rRNAs is critical for regular, functional ribosome assembly and translation to function normally. Liao (1999) concludes that "a possible biological function of concerted evolution is to maintain homogeneous gene copies in a family so that homogeneous transcripts can be produced." However, concerted evolution is thought to be a slow process over numerous generations. Hybrids would seem likely to be heterozygous for both parents nrDNA. Thus, nrDNA (ITS) has often been used for the analysis of hybridization.

There have been several reports where nrDNA in hybrids (and backcrosses?) has been more like one of the parents than a hybrid (i. e., heterozygous at every informative site). Chaing et al. (2001) reported that in artificial hybrids between *Begonia aptera* (pollen) and *B. formosana* (maternal), nrDNA was predominantly like that of the maternal parent, *B. formosana*. This is disturbing because having equal parts of nrDNA from both parents is a principle that is critical for the classification of plants as hybrids, or backcrosses. Thus, the predominate similarity to the maternal parent, *B. formosana*, would lead one to erroneously conclude that the hybrid was a backcross.

Volkov et al. (1999) reported that one of the parental nrDNAs was eliminated in the allopolyploid genome of cultivated tobacco. Fukuoka et al. (1994) found that the nrDNA in γ-ray irradiated tetraploid rice was homogenized in a short time. These reports clearly cause concern about the use of nrDNA for the detection of hybrids and introgression. However, it is noteworthy, that they do supply examples of the asymmetrical inheritance between parents, favoring one of the parents in hybridization.

Artificial hybrids were made between *Armeria villosa* ssp. *longiaristata* and *A. colorata*, then examined the inheritance of nrDNA in F_1 and F_2 generations (Aguilar et al. 1999). They found the expected additive pattern in polymorphisms for five of the six variable sites in F_1 plants. However, in the F_2 generation, there was a bias towards one parent (*A. colorata*). Backcrosses showed homogenization toward the recurrent parent for five of the six polymorphic sites to the recurrent parent. This asymmetrical inheritance of nrDNA clearly skewed the pattern such that backcrosses might be erroneously interpreted.

Introgression in *Mitella* was studied using nrDNA ITS and ETS, and cpDNA and found that cpDNA revealed the most introgression, ITS regions showed a moderate amount of introgression and the ETS region gave no evidence of introgression (Okuyama et al. 2005). They concluded that non-uniform concerted evolution between the ETS region and ITS regions explained these different patterns of introgression.

These reports clearly cause concern about the use of nrDNA for the detection of hybrids and introgression due to concerted evolution (Liao, 1999; Okuyama et al. 2005), maternally influenced inheritance (Chaing et al. 2001), and the exclusion of one parent's nrDNA in allopolyploid tobacco (Volkov et al. 1999). Each of the mechanisms for the homogenizing heterozygous nrDNA may explain the abnormalities of inheritance of nrDNA in hybrids and they provide mechanisms helpful in explaining cases of chloroplast capture in taxa derived by ancient hybridization (Adams, Schwarzbach and Tashev, 2016).

Due to the occasional asymmetrical inheritance of nrDNA (see above), there has recently been an expansion in the utilization of Single Copy Nuclear Genes (SCNGs), although in the Cupressaceae, there are few studies using SCNGs. A great example is that of Moreno-Letelier, Mastretta-Yanes and Barraclough (2014) who used six SCNGs proved useful in a study lineage divergence in *Juniperus blancoi*.

Adams (2015a, b) found, in field studies of *J. maritima* R. P. Adams x *J. scopulorum* Sarg. hybridization, that nrDNA identified 15 hybrids, whereas, maldehy, a single copy nuclear gene (SCNG), detected 25 hybrids. The nrDNA data frequently appeared to be the same as one of the parents, whereas the SCNG (maldehy) was heterozygous at both (2) informative sites, indicating the plant(s) were of hybrid origin. These studies Adams (2015a, b) were in natural populations, so it is often difficult to be completely confident that hybrids are being analyzed (as opposed to backcrosses, or F_2 plants). That factor has led us to examine inheritance of single copy nuclear genes (SCNGs) from the cypress cross analyzed by Adams, Miller and Low (2016).

In the Cupressaceae, breeding programs are rare, so the existence of parents and artificial (verified) hybrids is an important resource for studies on inheritance. Scion Research Institute, Rotorua, New Zealand has a breeding program that involves crossing *Cupressus* and *Hesperocyparis* species. The breeding program afforded an unusual opportunity to examine the inheritance of SCNGs in hybrids in the Cupressaceae. The purpose of this paper is to report on the inheritance of SCNGs in artificial hybrids of *H. arizonica* x *H. macrocarpa* and determine if their inheritance (as heterozygous in hybrids) validates their use in determining hybrids in natural populations.

MATERIALS AND METHODS

Plant material: Crosses were made at the Scion Research Institute, Rotorua, New Zealand using pollen of *H. arizonica* (2003.017) onto receptive seed cones of *H. macrocarpa* (896.752). Seedlings were obtained and greenhouse grown to 50-80 cm, then field planted. Leaf samples were taken after approximately one year in the field (plants about 1 m tall). Parents: *Adams 14854 H. arizonica* (2003.017), *Adams 14856 H. macrocarpa* (896.752), (leaves in silica gel), eighteen (18) Hybrids (leaves in silica gel) (lab accession #): *Adams 14914 - Adams 14931*.

One gram (fresh weight) of the foliage was placed in 20 g of activated silica gel and transported to the lab, thence stored at -20° C until the DNA was extracted. DNA was extracted from juniper leaves by use of a Qiagen mini-plant kit (Qiagen, Valencia, CA) as per manufacturer's instructions. Amplifications were performed in 30 µl reactions using 6 ng of genomic DNA, 1.5 units Epi-Centre Fail-Safe Taq polymerase, 15 µl 2x buffer E (petN-psbM) or K (nrDNA) (final concentration: 50 mM KCl, 50 mM Tris-HCl (pH 8.3), 200 µM each dNTP, plus Epi-Centre proprietary enhancers with 1.5 - 3.5 mM $MgCl_2$ according to the buffer used), 1.8 µM each primer. Nine single copy nuclear genes (SCNGs): *LHCA*4 (type IV chlorophyll binding protein), *maldehy* (malate dehydrogenase), *myb* (Myb transcription factor), *ABI3* (ABI3-interacting protein gene, *4CL* (4-coumarate CoA ligase), *CnAIP2* (*Callitropsis nootkatensis* abscisic acid-insensitive 2), *cc13333* (GTP binding protein gene), *chs* (chalcone synthase) and *hsp* (heat shock protein) (Adams et al. 2009, Moreno-Letelier et al. 2014, Zheng, et al. 2013) were sequenced for each of the parents (*H. arizonica* (2003.017; *H. macrocarpa* (896.752) to determine if they were informative in distinguishing the parents. Three SCNGs (*4CL, CnAIP3* and *myb*) were found to be potentially informative and these were sequenced for each of the18 hybrids.

The PCR reaction was subjected to purification by agarose gel electrophoresis. In each case, the band was excised and purified using a Qiagen QIAquick gel extraction kit (Qiagen, Valencia, CA). The gel purified DNA band with the appropriate sequencing primer was sent to McLab Inc. (San Francisco) for sequencing. Sequences for both strands were edited and a consensus sequence was produced using Chromas, version 2.31 (Technelysium Pty Ltd.).

RESULTS AND DISCUSSION

Sequencing myb of the parents (*H. arizonica* (2003.017; *H. macrocarpa* (896.752) revealed 5 sites with some heterozygosity: 89, 261, 338, 748, and 849. However, only two sites (89, 261) were

homozygous in both parents (89 CC TT; 261 GG, AA, Table 1) and thus, potentially useful for the detection of hybrids in natural populations. Each of the 18 artificial hybrids was heterozygous at loci 89 and 261 (Table 1) and these myb loci are useful in the detection of natural hybrids between *H. arizonica* and *H. macrocarpa* **if** analyses of *H. arizonica* and *H. macrocarpa* in the natural population being studied proved these taxa are always homozygous at sites 89 and 261. Our study of this cross indicates, but does not prove that myb, sites 89 and 261 would be useful in the study of a natural population (it might be noted that these two species are not sympatric in nature, so the discussion is somewhat academic).

The inheritance of the alleles at the other 3 sites (338, 748, 849) is interesting to examine. Only 4 of the 8 possible pollen haplotypes were present among the 18 hybrids (Table 1). The hybrids were found to be of 4 genotypes (Table 1, group 1: CT, GA, GG, CC, TC, with 9 plants; group 2: CT, GA, AG, AC, CC, with 6 plants; group 3: CT, GA, GG, CC, CC, with 2 plants; and group 4: CT, GA, AG, CC, TC, with 1 plant).

Group 1 pollen haplotype CGGCT was found in 9 hybrids, and group 2 pollen haplotype CGAAC was in 6 hybrids. It seems likely that these haplotypes are on 2 different chromosomes of *H. arizonica* (the male parent). Pollen haplotype, CGGCC, (Gp. 3, Table 1) was present in only 2 hybrids, and appears to be the result of cross-over between CGGC**T** and CGAA**C** between sites 748 and 849 (100 bp distance) to produce the CGGC**C** haplotype.

The haplotype, CGACT, (Gp. 4, Table 1) was found in only 1 hybrid. It appears be the product of cross-over between CGA**AC** and CGG**CT** at sites 338 and 749 (410 bp) to produce the CGACT haplotype.

It should be noted that there was only one egg haplotype (TAGCC), because *H. macrocarpa* was homozygous at all 5 sites (TT, AA, GG, CC, CC, Table 1) and this facilitated the analyses of the pollen haplotypes.

Sequencing 4CL of the parents discovered 8 sites with some heterozygosity: 507, 529, 531, 533, 591, 612, 638 and 644. Only one site (591) was homozygous in both parents (591: TT, CC, Table 2) and likely useful for the detection of hybrids. Each of the 18 artificial hybrids was heterozygous at locus 591 (Table 2). Thus, the 4CL locus may be useful in the detection of natural hybrids between *H. arizonica* and *H. macrocarpa* **if** analyses of *H. arizonica* and *H. macrocarpa* in the natural population being studied proved these taxa are always homozygous at site 591.

The inheritance of the alleles at the other 7 sites (507, 529, 531, 533, 612,638, 644) is interesting to examine. Notice how physically close these sites are (529, 531, 533; and 638, 644). Only 2 of the 8 possible pollen haplotypes were present among the 18 hybrids (Table 2). The hybrids were found to be of 4 genotypes (Table 2, group 1: AA, CC, CT, AA, TC, CG, AG, AA, with 9 plants; group 2: TA, CT, CC, GA, TC, CC, AA, GA, with 5 plants; group 3: AA, CT, CC, AA, TC, CC, AA, AA, with 3 plants; and group 4: TA, CC, CT, GA, TC, CG, AG, GA, with 1 plant).

Group 1 pollen haplotype (ACCATCAA) was found in 12 hybrids, and pollen haplotype TCCGTCAG (Group 2) was in 6 hybrids. It seems likely that these haplotypes are on 2 different chromosomes of *H. macrocarpa* (the female parent).

Two egg haplotypes were found: Group 1 egg haplotype (ACTACGGA in 10 hybrids, and egg haplotype ATCACCAA (Group 2) was in 8 hybrids, implying these haplotypes are on 2 chromosomes of *H. macrocarpa* (the female parent, Table 2).

Sequencing CnAIP2 of the parents (*H. arizonica* (2003.017; *H. macrocarpa* (896.752) revealed two sites with some heterozygosity: 301 and 554, but neither of these were useful for hybrid detection (Table 3).

The inheritance of the alleles at the two sites (301, 554) is interesting to examine. Only 2 of the 4 possible pollen haplotypes and 2 of the 4 egg possible haplotypes were present among the 18 hybrids (Table 3). The hybrids were found to be of 4 genotypes (Table 3, group 1: AA, AA, in 6 plants; group 2: AC, AG, in 6 plants; group 3:AC, AA, in 4 plants; and group 4: AA, AG in 2 plants).

Group 1 pollen haplotype (AA) was found in 10 hybrids, and pollen haplotype AG (Group 2) was in 8 hybrids, implying these haplotypes are on 2 chromosomes of *H. arizonica* (the male parent).

Two egg haplotypes were found: Group 1 egg haplotype (AA) in 8 hybrids, and egg haplotype CA (Group 2) was in 10 hybrids, implying these haplotypes are on 2 chromosomes of *H. macrocarpa* (the female parent).

In summary, the survey of 9 SCNGs using only two parents (*H. arizonica, H. macrocarpa*), yielded only 3 candidate genes, of which only 2 proved useful. These 2 genes had only three informative sites (myb, 2; 4CL, 1). However, these three sites showed perfectly clean chromatograms that were always heterozygous in all 18 hybrids. It is possible that the other 6 'SCNGs' were, at least in this instance, multi-copy genes.

Two novel pollen haplotypes were discovered in myb in *H. arizonica* pollen. Haplotype CGGCC, present in 2 hybrids, appears to from a cross-over between sites 748 and 849 (100 bp gap). The second haplotype, CGACT, in 1 plant, seems to have arisen by a cross-over between sites 338 and 748 (409 bp gap).

In *H. macrocarpa*, gene 4CL had a deletion (5 bp) beginning at position 90 and a deletion at 151 (70 bp) and *H. arizonica* had a deletion (49 bp) at 323 that led slippage in the hybrid's sequences and un-callable bases. This can be addressed by NextGen sequencing.

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Table1. Variable myb sites in hybrids between *H. arizonica* (14854, 2000.75,0.2517) x *H. macrocarpa* (14858, 896.752) cross. Parent differ at 8 sites. Site numbering is from the 5' end. Hybrids all full-sibs. Pollen haplotypes not found: CGGAT,CGACC, CGAAT, CGGAC.

5 sites analyzed	89[1]	261[2]	338[3]	748[4]	849[5]	
pollen haplotypes found:	*arizonica* genotype (male)					Frequency by group
	CC	GG	GA	CA	TC	
CGGCT	C	G	G	C	T	Gp. 1(9)
CGAAC	C	G	A	A	C	Gp. 2(6)
CGGCC	C	G	G	C	C	Gp. 3(2)
CGACT	C	G	A	C	T	Gp. 4(1)
	macrocarpa genotype (female)					
egg haplotype	TT	AA	GG	CC	CC	
TAGCC	T	A	G	C	C	All Gps.18/18
Groups found						
Gp. 1, pollen	C	G	G	C	T	CGGCT
egg	T	A	G	C	C	TAGCC
14914 Gp. 1(9)	CT	GA	GG	CC	TC	2/5 # homozygous
14915	CT	GA	GG	CC	TC	2/5
14918	CT	GA	GG	CC	TC	2/5
14919	CT	GA	GG	CC	TC	2/5
14920	CT	GA	GG	CC	TC	2/5
14925	CT	GA	GG	CC	TC	2/5
14926	CT	GA	GG	CC	TC	2/5
14928	CT	GA	GG	CC	TC	2/5
14931	CT	GA	GG	CC	TC	2/5
Gp. 2, pollen	C	G	A	A	C	CGAAC
egg	T	A	G	C	C	TAGCC
14917 Gp. 2(6)	CT	GA	AG	AC	CC	1/5
14921	CT	GA	AG	AC	CC	1/5
14923	CT	GA	AG	AC	CC	1/5
14927	CT	GA	AG	AC	CC	1/5
14929	CT	GA	AG	AC	CC	1/5
14930	CT	GA	AG	AC	CC	1/5
Gp. 3. pollen	C	G	G	C	C	CGGCC
egg	T	A	G	C	C	TAGCC
14922 Gp. 3(2)	CT	GA	GG	CC	CC	3/5
14924	CT	GA	GG	CC	CC	3/5
Gp. 4.pollen	C	G	A	C	T	CGACT
egg	T	A	G	C	C	TAGCC
14916 Gp. 4(1)	CT	GA	AG	CC	TC	1/5
summary of genotypes	all CT homoz y	all GA homoz y	11 GG, 7 AG	12 CC 6 AC	8 CC 10 CT	
heterozygous/ total	18/ 18	18/ 18	7/ 18	6/ 18	10/ 18	

[1] left of GCTATTAAG, [2] left of GCGATTTTA, [3] left of CCGGGGTCA
[4] left of CGGAGCGTT, [5] left of CCCCTTTTC

Table 2. Variable 4CL sites in hybrids between *H. arizonica* (14854, 2000.75,0.2517) x *H. macrocarpa* (14858, 896.752) cross that differ at 8 sites. Site numbering is from the 5' end.

8 sites analyzed	507[1]	529[2]	531[3]	533[4]	591[5]	612[6]	638[7]	644[8]	
pollen haplotypes found:	*arizonica* genotype (male)								Frequency by groups
	A/T	C/C	C/C	A/G	T/T	C/C	A/A	A/G	
ACCATCAA	A	C	C	A	T	C	A	A	Gp 1(9), Gp 3(3)
TCCGTCAG	T	C	C	G	T	C	A	G	Gp 2(5), Gp 4(1)
egg haplotypes found:	*macrocarpa* genotype (female)								
	A/A	C/C	C/T	A/A	C/C	C/G	A/G	A/A	
ACTACGGA	A	C	T	A	C	G	G	A	Gp 1(9), Gp4(1)
ATCACCAA	A	T	C	A	C	C	A	A	Gp 2(5), Gp 3(3)
Groups found									
Gp 1. pollen	A	C	C	A	T	C	A	A	ACCATCAA
egg	A	C	T	A	C	G	G	A	ACTACGGA
14914 Gp. 1(9)	AA	CC	CT	AA	TC	CG	AG	AA	4/8 # homozygous
14919	AA	CC	CT	AA	TC	CG	AG	AA	4/8
14920	AA	CC	CT	AA	TC	CG	AG	AA	4/8
14921	AA	CC	CT	AA	TC	CG	AG	AA	4/8
14923	AA	CC	CT	AA	TC	CG	AG	AA	4/8
14928	AA	CC	CT	AA	TC	CG	AG	AA	4/8
14929	AA	CC	CT	AA	TC	CG	AG	AA	4/8
14930	AA	CC	CT	AA	TC	CG	AG	AA	4/8
14931	AA	CC	CT	AA	TC	CG	AG	AA	4/8
Gp 2. pollen	T	C	C	G	T	C	A	G	TCCGTCAG
egg	A	T	C	A	C	C	A	A	ATCACCAA
14915 Gp. 2(5)	TA	CT	CC	GA	TC	CC	AA	GA	3/8
14922	TA	CT	CC	GA	TC	CC	AA	GA	3/8
14917	TA	CT	CC	GA	TC	CC	AA	GA	3/8
14926	TA	CT	CC	GA	TC	CC	AA	GA	3/8
14927	TA	CT	CC	GA	TC	CC	AA	GA	3/8
Gp 3. pollen	A	C	C	A	T	C	A	A	ACCATCAA
egg	A	T	C	A	C	C	A	A	ATCACCAA
14916 Gp. 3(3)	AA	CT	CC	AA	TC	CC	AA	AA	6/8
14918	AA	CT	CC	AA	TC	CC	AA	AA	6/8
14924	AA	CT	CC	AA	TC	CC	AA	AA	6/8
Gp 4. pollen	T	C	C	G	T	C	A	G	TCCGTCAG
egg	A	C	T	A	C	G	G	A	ACTACGGA
14925 Gp. 4(1)	TA	CC	CT	GA	TC	CG	AG	GA	1/8
summary of genotypes	11 AA 7 AT	10 CC 8 CT	10 CT 8 CC	12 AA 6 AG	all TC homo zy	10 CG 8 CC	10 AG 8 AA	12 AA 6 AG	
# homozygous/ heterozygous	11/ 7	10/ 8	8/ 10	12/ 6	0/ 18	8/ 10	8/ 10	12/ 6	

[1] left of CATTCATTA, [2] right of GAGTAGTT, [3] left of TACACAATT, [4] left of CACAATTCG, [5] left of TCTAAAAA, [6] left of TAGAACAAT, [7] right of CTTTCAAC, [8] left of GTACCCTTT,

Table 3. Variable CnAIP2 sites in hybrids between *H. arizonica* (14854, 2000.75,0.2517) x *H. macrocarpa* (14858, 896.752) cross which differ at 2 sites. Site numbering is from the 5' end.

2 sites analyzed	301[1]	554[2]	
	arizonica genotype (male)		
pollen haplotypes	AA	AG	freq. by group
AA	A	A	Gp. 1 (6), Gp. 3 (4)
AG	A	G	Gp. 2 (6), Gp. 4 (2)
	macrocarpa genotype (female)		
egg haplotypes	AC	AA	
AA	A	A	Gp. 1 (6), Gp. 4(2)
CA	C	A	Gp. 2 (6), Gp. 3 (4)
Groups found			
Gp. 1. pollen	A	A	AA
egg	A	A	AA
14914 Gp 1. (6)	AA	AA	4/4
14920	AA	AA	4/4
14924	AA	AA	4/4
14925	AA	AA	4/4
14930	AA	AA	4/4
14931	AA	AA	4/4
Gp. 2. pollen	A	G	AG
egg	C	A	CA
14915 Gp 2 (6)	AC	AG	2/4
14919	AC	AG	2/4
14921	AC	AG	2/4
14923	AC	AG	2/4
14922	AC	AG	2/4
14928	AC	AG	2/4
Gp. 3. pollen	A	A	AA
egg	C	A	CA
14917 Gp 3. (4)	AC	AA	1/4
14918	AC	AA	1/4
14927	AC	AA	1/4
14929	AC	AA	1/4
Gp. 4. pollen	A	G	AG
egg	A	A	AA
14916 Gp 4. (2)	AA	AG	1/4
14926	AA	AG	1/4
summary of genotypes	8 AA 10 AC	10 AA 8 AG	
homozygous/ heterozygous	8/ 10	10/ 8	

[1] left of ATGTGCTT, [2] left of CAGCATCT

Palynology of different populations of *Juniperus polycarpos* complex in Iran

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ABSTRACT

Analyses of *Juniperus* in Iran, which have been treated as *Juniperus excelsa* in Iran, using morphology, isoenzyme, terpenoids and DNA sequences, revealed that two cryptic, genetically distinct but morphologically almost identical species are distributed in the country. These two species are *J. polycarpos* with two varieties distributed in N and *J. seravschanica* distributed in SE Iran. The most important character in diagnosing these species is the thickness of the ultimate branchlets. As micro morphological characters, all pollen grains were spherical, monoporate with echinnate orbicules as sculpture elements. Based on the pollen morphology we can observe the shortest pollen grains orbicules echinae and, relatively, the biggest pollen grains and pores in populations from S Iran as *J. seravschanica*. Published on-line www.phytologia.org *Phytologia 101(1): 67-73 (March 21, 2019)*. ISSN 030319430.

KEY WORDS: cryptic species, pollen grains, micro morphological character, *J. polycarpos* complex, *J. seravschanica,* Cupressaceae, SEM, Iran.

The genus *Juniperus* (Cupressaceae) is composed of approximately 75 species in 3 monophyletic sections (Mao et al. 2010, Adams and Schwarzbach 2013): *Caryocedrus* with one species, *Juniperus*, with 14 species and *sabina* with 60 species. Among these species, *J. excelsa* M. Bieb of sect. *Sabina* and relative taxa (referred here as *J. excelsa* complex) form a taxonomically difficult group, as the borders between taxa cannot be sharply defined by morphology. According to the latest study, this complex consists of three morphologically very similar species as: *J. excelsa*., *J. polycarpos* K. Koch. (var. *polycarpos* Linnaea and var. *turcomanica* R.P. Adams) and *J. seravschanica* Kom. (Hojjati and Adams unpublished) In Iran *J. polycarpos* is distributed in N with two varieties: *polycarpos* and *turcomanica*, *J. seravschanica* is distributed in SE and hybrid samples are distributed in SW of the country and there is no *J. excelsa* (Hojjati et al. 2018).

However there are different treatments on this group depends on the taxonomic value that each botanist arbitrarily attributed to these taxa (Marschal von Bieberstein, 1800, 1808; Koch, 1849; Boissier, 1884; Fedtschenko et al., 1932; Komarov, 1932; Riedel, 1968; Farjon, 1992; Assadi, 1998; Adams, 1999, 2014). Some populations of the Iran *J. excelsa* complex have been studied.

Based on isoenzyme data, Hojjati *et al.* (2009) recognized 3 major clusters as *J. polycarpos* var. *polycarpos*, *J. polycarpos* var. *turcomanica* and *J. seravschanica* of this complex in Iran. Adams and Shanjani (2011), using DNA sequence data, showed the juniper from the Elburz Mtns. to be typical *J. polycarpos* not *J. excelsa*. Subsequently, Adams and Hojjati (2012) and Adams *et al.* (2014) employing four DNA regions (nrDNA, *pet*N-*psb*M, *trn*D-*trn*T and *trn*S-*trn*G, 3,705 nucleotide site) showed that the samples from NW Iran are *J. polycarpos* and samples from NE Iran are clearly *J. polycarpos* var. *turcomanica*, as are the samples from Fasa in SW Iran. The samples from nearby southcentral Iran (Khabr protected area) are part of a clade with *J. seravschanica* (Komarov) Kitamura.

Several studies on the pollen morphology of members of Cupressceae have been published. An initial overview of the pollen morphology of this family was provided by Erdtman (1965). Hyde and Adams (1958), Kapp (1969), Bassett et al. (1978) and Ciampolini and Cresti (1981) reported that pollen grains of *Juniperus* are inapeturate, but Nilsson et al. (1977), Moor and Webb (1978), Lewis et al. (1983) and Bortenschlager (1990) described monoporate pollen grains in *Juniperus*. With respect to pollen sculpture, it has been generally stated that the surface of pollen grains is irregularly scabrate and is covered by irregularly arranged orbicules (Huiho and Sziklai, 1973; Pocknall, 1981; Bortenschlager, 1990; Yu, 1997). These studies indicate high similarity and uniformity of pollen structure among different taxa of Cupressaceae.

The present study compared *J. polycarpos* and *J. seravschanica* micromorphologically to determine if they have any differences in their pollen exine.

MATERIALS AND METHODS

Twelve populations of *J. polycarpos* complex in Iran were sampled. The voucher specimens were deposited in the Central Herbarium of Tehran University, TUH. Population name, location, herbarium number, longitude, latitude and altitude for each population were listed in Table 1.

For the micro morphological study, pollen grains of 12 populations of *J. polycarpos* complex in Iran were studied by light microscope (LM) and scanning electron microscope (SEM). The pollen samples were obtained from both fresh and dried herbarium specimens and then prepared following rhodaniden method described by Tatzreiter (1985). In comparison with the Erdtman's acetolysis method that splits pollen grains into two halves, the gentler rhodanid method prevents the occurrence of artifacts in pollen grains so that they remain almost without exception in their original spherical form. In rhodaniden method, briefly, we used two solutions: solution A that is composed of HCl, $MgCl_2$, $MgSO_4$, $AlCl_3$ and $AlPO_4$ and solution B that contains KSCN or NaSCN. The prepared materials provided specimens for investigations on LM as well as SEM. Slides for LM were studied and photographed. The diameter of spherical pollen grains and exine thickness were measured and with aid of a ×100 eyepieces. Measurement of pollen grains was based on 30 grains per sample. For SEM study the pollen grains were put on stubs and sputter coated with approximately 25 nm of gold-palladium (Au-Pd) alloy and then scanned in a Vega SEM model VG2080573IR at 15 kV.

RESULTS AND DISCUSSION

Nine quantitative and qualitative pollen morphological characters studied in 12 populations are listed in Table 2. All pollen grains surveyed were spherical and monoporate so that a circular pore situated at the distal pole. The diameter of this pore was consistently less than 2 µm. The exine elements were spherical shaped orbicules that were isolated or in larger or smaller groups. It appeared that the arrangement of these elements was generally looser at the proximal pole. Their surfaces were themselves occupied with small echinae. Figures 1 (1-12) show some photographs of the studied pollen grains. The mentioned features utilized for pollen morphology in this study were as below. Pollen diameter ranged from 26.2 µm in Lushan1 population to 31 µm in Fasa and showed the highest amounts in most of the southern populations. Exine thickness was mostly stable at 1 µm but was 1.2 µm in Qushchi population. Pollen grain pore diameter varied from 0.5 µm in Shahmirzad to 1.8 µm in Genu population. In this case all of the southern populations showed higher values than most of the other populations. Sculpture regulation as a qualitative character was mostly stable with low variation between populations, the same pattern was also found for sculpture density. Sculpture amount had 3 values for variations. Orbicules diameter ranged from 0.7 µm to 1 µm in Fasa population. Annulus existed around the pollen grains pore only in a few populations (Fig. 8). Orbicules echinae length differs among populations studied here so that it was middle in northwestern populations, high in north and northeastern ones and low in southern

populations (Figs. 3, 6, 9 and 12). Based on the pollen morphology we can observe the shortest pollen grains orbicules echinae, relatively, the biggest pollen grains and pores in southern populations.

Table 1. Location of populations of *Juniperus polycarpos* complex studied in Iran.

Population name	Species	Location	Herbarium number	Lat., Long.	Elev (m)
Lushan1	*J. polycarpos* var. *polycarpos*	N Iran, Prov. Gilan, Lushan to Jirandeh, 15 km to Jirandeh.	33606	36 º 40′ N, 49º 38′ E	1100-1120
Lushan2	*J. polycarpos* var. *polycarpos*	N Iran, Prov. Gilan, 5 km after Jirandeh towards Yeilaq and Amarlu.	33607	86º 40′ N, 49º 42′ E	1670-1690
Hashtjin	*J. polycarpos* var. *polycarpos*	N Iran, Prov. Ardebil, 16 km towards Hashtjin after Khalkhal.	33608	37º 26′ N, 48º 24′ E	1590-1610
Qushchi	*J. p.* var. *polycarpos*	N Iran, Prov. Azerbaijan, 28 km to Salmace after Orumiee.	33609	38º 0.1′ N, 44º 57′ E	1730-1800
Shahmirzad	*J. polycarpos* var. *turcomanica*	N Iran, Prov. Khorasan, 15 km after Shahmirzad towards Fooladmahaleh.	33610	35º 50′ N, 53º 26′ E	2422
Bajgiran	*J. polycarpos* var. *turcomanica*	N Iran, Prov. Khorasan. 35 km to Bajgiran.	33611	37º 25′ N, 58º 32′ E	1868
Golestan	*J. polycarpos* var. *turcomanica*	N Iran, Prov. Gorgan, Golestan National Park, Sharleq, 9 km towards Azadshahr.	33612	37º 19′ N, 56º 2′ E	1055
Balade	*J. polycarpos* var. *turcomanica*	N Iran, Prov. Mazandaran, 10 km after Baladeh towards Kujur.	33613	36º 14′ N, 51º 50′ E	2924
Fasa	*J. polycarpos* X *J. seravschanica*	S Iran, Prov. Fars, 30 km after Fasa towards Neiriz.	33614	29º 9′N, 53º 40′ E	1607
Kuhbanan	*J. seravschanica*	S Iran, Prov. Kerman, Kuh-e Bajgen, 55 km towards Kuhbanan, Dolatabad village.	33615	31º 28′ N, 55º 52′ E	2091
Khabr	*J. seravschanica*	of Iran, Prov. Kerman, Kuh-e Khabr.	33616	28º 51′ N, 56º 22′ E	2418
Genu	*J. seravschanica*	S Iran, Prov. Kerman, Kuh-e Genu	33618	27º 24′ N, 56º 11′ E	1673

Table 2. Nine quantitative and qualitative micro morphological characters studied in 12 populations of Iran *Juniperus polycarpos* complex. A= irregular B= relatively regular C= mostly isolated D= mostly clustered E= low F= middle G= high H= presence I= absence J= short K= medium L= long

Populatiion, taxon	Characters								
	Pollen grain diam. (µm)	Exine thickness (µm)	Pore diam. (µm)	Orbicules regulation	Orbi-cules density	Orbi-cules amount	Orbi-cules diam. (µm)	Existence of annulus around the pore	Orbi-cules echinae length
Lushan1 *J. p.* var. *polycarpos*	24.0 26.2 29.0	1.0	0.8	B	C	F	0.8	I	K
Lushan2 *J. p.* var. *polycarpos*	25.0 27.0 30.0	1.0	1.2	A	D	F	0.8	I	K
Hashtjin *J. p.* var. *polycarpos*	26.0 27.6 29.0	1.0	1.0	A	D	G	0.7	I	K
Qushchi *J. p.* var. *polycarpos*	27.0 29.0 31.0	1.0 1.2 2.0	1.1	A	D	G	0.9	I	K
Shahmirzad *J. p.* var. *turcomanica*	26.0 28.0 30.0	1.0 1.1 2.0	0.5	A	D	E	0.7	I	L
Bajgiran *J. p.* var. *turcomanica*	26.0 29.3 33.0	1.0	1.4	A	D	G	0.7	I	L
Golestan *J. p.* var. *turcomanica*	27.0 28.6 32.0	1.0	1.2	B	C	G	0.9	H	L
Balade *J. p.* var. *turcomanica*	26.0 26.8 29.0	1.0	1.7	A	D	E	0.7	H	L
Fasa *J. polycarpos X J. seravschanica*	27.0 31.0 34.0	1.0	1.4	A	D	E	1.0	H	J
Kuhbanan *J. seravschanica*	27.0 30.0 32.0	1.0	1.5	A	D	G	0.8	I	J
Khabr *J. seravschanica*	26.0 29.0 32.0	1.0	1.6	A	D	G	0.8	I	J
Genu *J. seravschanica*	26.0 29.4 33.0	1.0	1.8	A	D	E	0.8	H	J

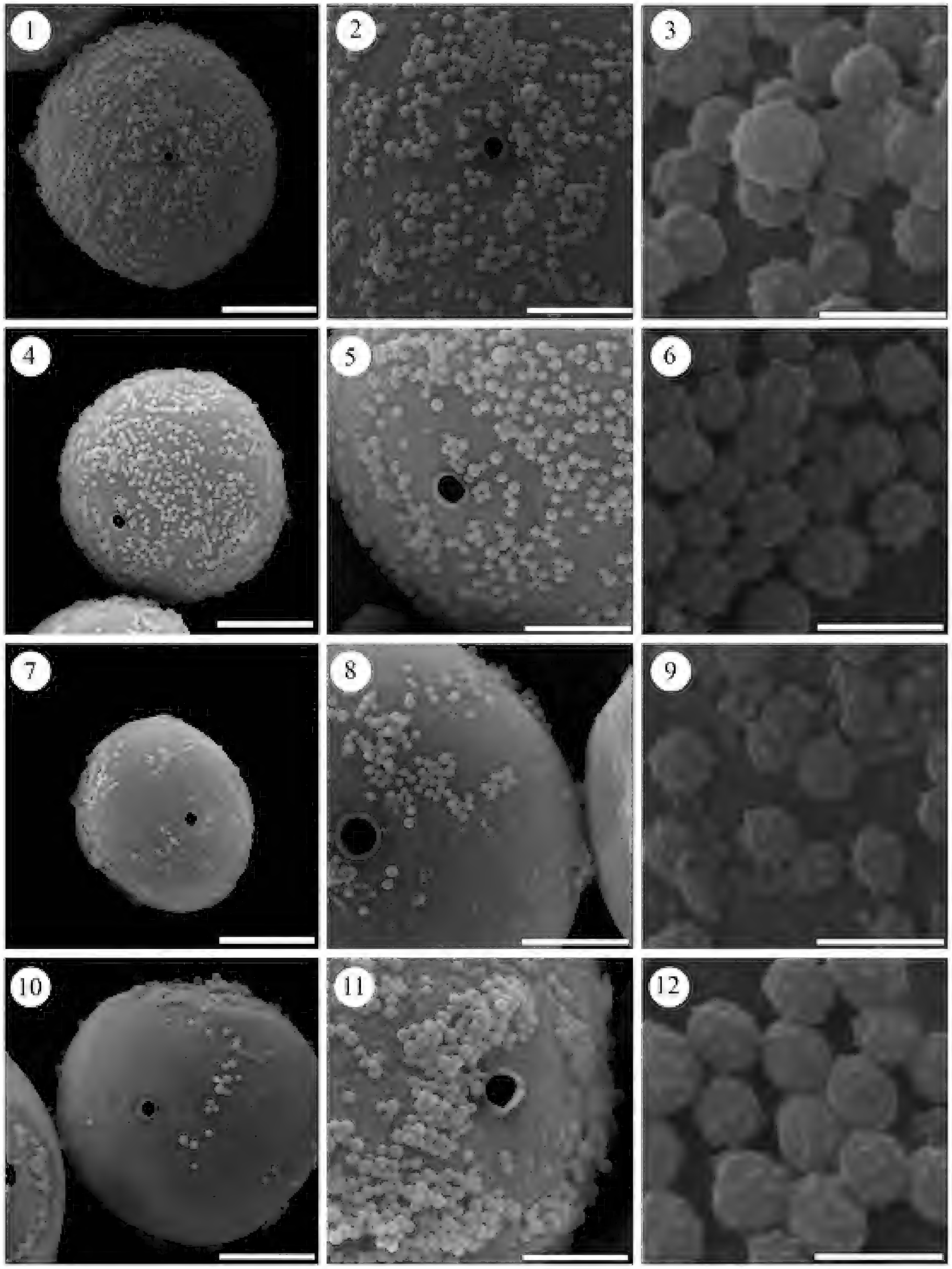

Fig.1. 1-12, SEM micrographs of pollen grains in some of *Juniperus polycarpos* complex Iran populations. Figs. 1 and 2, Pollen grain from Qushchi population. Fig. 3, Pollen orbicules echinae from Hashtjin. Figs. 4 and 5, Pollen grain from Golestan. Fig. 6, Pollen orbicules echinae from Bajgiran. Figs. 7 and 8, Pollen grain from Balade. Fig. 9, Pollen orbicules echinae from Balade. Fig. 10, Pollen grain from Fasa. Fig. 11, Pollen grain from Khabr. Fig. 12, Pollen orbicules echinae from Genu. Bar scale 1, 4, 7, 10=10 µm, 2, 5, 8, 11=5 µm and 3, 6, 9, 12=1 µm.

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Composition of the leaf volatile terpenoids of *Pinus mugo* Turra from Bulgaria compared with oils from other regions.

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ABSTRACT

The volatile leaf oil of *Pinus mugo,* Bulgaria, is composed of 66 components (with numerous additional trace compounds) with large amounts of δ-3-carene (24.6%), β-phellandrene (16.7%), α-pinene (11.0%), and lesser concentrations of β-pinene (2.7), terpinolene (3.9), bornyl acetate (4.2), (E)-caryophyllene (5.3), germacrene D (1.7), α-cadinol (1.1), palustral (1.0). The leaf volatile oil of *P. mugo* from Bulgaria is quite similar to oils from other Balkan populations as per the literature. However, an analysis from *P. mugo* oil from central Italy was quite different from *P. mugo* from the Balkans, indicating the need for additional research on *P. uncinata* leaf oils. Published on-line www.phytologia.org *Phytologia 101(1): 74-80 (March 21, 2019).* ISSN 030319430.

KEY WORDS: *Pinus mugo* subsp. *mugo,* Bulgaria, volatile leaf oil, terpenes, composition, *Pinus mugo subsp. uncinata* (*P. uncinata*).

Pinus mugo Turra has been treated as composed of two subspecies: subsp. *mugo* and subsp. *uncinata* (Raymond) Domin (Chrsitensen 1987) or as two species: *P. mugo* and *P. uncinata* (Richardson 1998). *Pinus mugo* subsp. *mugo* is distributed in northern Italy into the Balkans and in eastern Europe and *P. uncinata* is distributed from the Alps, southwestward into France and Spain with hybridization between the subspecies in the zone of contact in the central European alps (Richardson, 1998).

The volatile leaf essential oils (terpenes) of *P. mugo* have been analyzed from Italy (Bambagiotii and Vincieri 1972; Venditti et al. 2013, Table 1), Macedonia (Karapandzova et al. 2011; Slovenia (Bojovic et al. 2016, Table 1); Sar Mountains (Mitic et al. 2017, Table 1) and cultivated, Cambridge Botanic Gardens (Ioannou et al. 2014). The volatile oil of P. uncinata (*P. mugo* subsp. *uncinata*) has been reported from central Italy (Venditti, et al. 2013) and from cultivated trees in Poland (Celinski et al. 2015, Bonikowski et al. 2015).

Several other papers cited *P. mugo* volatile analyses, but samples were obtained from cultivated materials of unknown source, and thus, not of interest in this study of geographical variation in *P. mugo* subsp. *mugo*.

MATERIALS AND METHODS

Leaf samples collected: *Pinus mugo* subsp. *mugo*, common at edge of a meadow, near forest by *Pinus peuce* and *P. heldrichii* with *Juniperus communis*. Bulgaria, 47° 45' 52.8" N, 23° 25' 22.6" E., 1838 m. Bulgaria, Coll. *Alex Tashev 1-5*, 12 June 2018, Lab Acc. *Robert P. Adams 15495, 15496, 15497, 15498, 15499*. Voucher specimens are deposited in the herbarium, Baylor University.

Gently dried leaves (100g, 40 - 45˚C) were steam distilled for 2 h using a circulatory Clevenger-type apparatus (Adams, 1991). The oil samples were concentrated (ether trap removed) with nitrogen and the samples stored at -20ºC until analyzed. The extracted leaves were oven dried (100ºC, 48 h) for determination of oil yields.

The oils were analyzed on a HP5971 MSD mass spectrometer, scan time 1/ sec., directly coupled to a HP 5890 gas chromatograph, using a J & W DB-5, 0.26 mm x 30 m, 0.25 micron coating thickness, fused silica capillary column (see Adams, 2007 for operating details). Identifications were made by library searches of the Adams volatile oil library (Adams, 2007), using the HP Chemstation library search routines, coupled with retention time data of authentic reference compounds. Note that limonene and β-phellandrene elute as a single peak on DB-5, but their amounts can be quantitated by the ratio of masses 68, 79 (limonene) and 77, 93 (β-phellandrene). Quantitation was by FID on an HP 5890 gas chromatograph using a J & W DB-5, 0.26 mm x 30 m, 0.25 micron coating thickness, fused silica capillary column using the HP Chemstation software.

RESULTS AND DISCUSSION

The composition of the volatile leaf oil of *Pinus mugo* subsp. *mugo* from Bulgaria is given in table 1. The volatile leaf oil contains large amounts of δ-3-carene (24.6%), β-phellandrene (16.7%), and α-pinene (11.0%), with moderate concentrations of β-pinene (2.7), terpinolene (3.9), bornyl acetate (4.2), (E)-caryophyllene (5.3), germacrene D (1.7), α-cadinol (1.1) and palustral (1.0). This compositional pattern seems common in the Balkan populations (Slovenia, Kosovo, Table 1). However, the putative *P. mugo* subsp. *mugo* from central Italy (Table 1, Venditti et al. 2013) has a very different terpene profile with δ-3-carene (0.8%), β-phellandrene (1.2%), and α-pinene (trace) being very small, as well as numerous other components being different (Table 1). This suggests the central Italy population may be *P. uncinata* (*P. mugo* subsp. *uncinata*). Recently, Boratynska et al. (Fig. 1, 2015) reported the natural ranges of *P. mugo* and *P. uncinata*, but subsequent morphological analyses revealed the central Italy *P. mugo* population to be *P. uncinata* (Fig. 5, Boratynska et al. 2015). Celinski et al. (2015), using head-space to analyze the oil of *P. uncinata* (cultivated in Poland, natural source not reported), so their results are not exactly compatible with steam distilled oils compositions (Table 1). However, coupled with the morphological analysis of Boratynska et al. (2015) that indicates the central Italy population is most like *P. uncinata*, it may be that the central Italy oil (Table 1) is that of *P. uncinata.* Additional research is needed to resolve this.

Variation among the individuals in the Bulgaria, *P. mugo* population (Table 2) is moderate with ranges of: δ-3-carene (19.8 - 28.2%), β-phellandrene (12.8 - 21.6%), α-pinene (7.3 - 17.1 %), β-pinene (1.7 - 3.3), terpinolene (3.1 - 4.6), bornyl acetate (1.6 - 8.9), (E)-caryophyllene (4.2 - 6.8), germacrene D (0.4 - 2.9), germacrene D-4-ol (1.2 - 3.3) and palustral (0.6 - 1.5). No chemotypes were apparent.

In conclusion, the leaf volatile oils of *P. mugo* from Bulgaria were quite similar to those from other Balkan populations reported in the literature (Table 1).

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Table 1. The leaf oil constituents of *Pinus mugo* from Bulgaria compared with other analyses on the volatile leaf oils. Compounds in bold face vary among locations. Compositional values less than 0.1% are denoted as traces (t). Unidentified components less than 0.5% are not reported. KI is the Kovat's Index using a linear calculation on DB-5 column. Note: *P. uncinata* analysis was based on head space volatiles (Celinski, et al. 2015), so it is not exactly compatible with the other steam distilled oils' compositions.

KI	compound	Bulgaria *15773* this study	Kosovo Oshlak Hajdari[1]	Kosovo Hajle Hajdari[1]	Sar Mtns. s. Kosovo Mitic[2]	Slovenia Julian Alps Bojovic[3]	Cambridge UK, Cult. Iannou[4]	*uncinata* central Italy Venditti[5]	*uncinata* cultivated Poland Celinski[6]
921	tricyclene	0.2	0.9	0.9	0.8	0.4 – 0.6	0.5	-	0.8
924	α-thujene	0.3	1.3	t	1.5	0.5 – 0.8	1.2	-	-
932	**α-pinene**	**11.0**	**17.0**	**19.9**	**18.0**	**12.9 -17.6**	**13.7**	**t**	**27.8**
946	**camphene**	**1.2**	**3.2**	**3.2**	**3.0**	**1.5 – 2.2**	**2.4**	**-**	**9.9**
969	sabinene	1.2	1.0	0.9	1.0	1.3 – 1.6	1.1	-	-
974	**β-pinene**	**2.7**	**5.5**	**2.8**	**4.1**	**2.7 - 8.7**	**2.1**	**0.1**	**-**
988	myrcene	3.1	2.6	2.4	2.2	2.9 – 12.2	6.9	0.1	8.9
1002	α-phellandrene	0.5	0.5	1.0	0.4	0.2 -0.6	-	t	0.2
1008	**δ-3-carene**	**24.6**	**17.7**	**27.9**	**21.3**	**13.0 – 27.0**	**9.9**	**0.8**	**t**
1014	α-terpinene	0.2	0.3	0.2	0.6	0.2 – 0.2	0.5	t	0.1
1020	p-cymene	0.2	0.2	0.3	0.1	0.1 – 0.2	-	t	-
1024	limonene	t	t?	t?	t?	t	t	t	7.0
1025	**β-phellandrene**	**16.7**	**5.9**	**3.8**	**7.6**	**14.5 – 18.0**	**2.6**	**1.2**	**7.6**
1044	(E)-β-ocimene	0.6	1.2	0.8	0.5	t	0.6	0.3	6.4
1054	γ-terpinene	0.4	0.5	0.4	0.7	0.3 – 0.5	0.4	0.1	0.2
1086	**terpinolene**	**3.9**	**4.3**	**2.8**	**5.5**	**3.0 - 3.4**	**3.8**	**0.5**	**0.8**
1095	linalool	-	0.2	t	0.2	-	-	0.3	-
1118	cis-p-menth-2-en-1-ol	0.1	-	-	-	t	-	0.3	-
1132	cis-limonene oxide	0.1	-	-	-	-	-	0.5	-
1136	trans-sabinol	0.1	-	-	-	-	-	-	-
1141	camphor	-	0.2	0.5	t	-	-	0.2	-
1145	camphene hydrate	-	-	-	t	-	-	0.3	-
1165	borneol	0.2	0.5	0.3	0.2	t	-	1.5	-
1174	**terpinen-4-ol**	**0.4**	**t**	**t**	**0.9**	**0.2 – 0.3**	**0.2**	**3.6**	**-**
1176	m-cymen-8-ol	0.2	t	t		-	-	0.5	-
1179	p-cymen-8-ol	0.3	t	t	t	-	-	1.6	-
1183	cryptone	-	-	-	-	t	-	0.4	-
1186	**α-terpineol**	**t**	**0.2**	**t**	**0.4**	**t**	**-**	**7.3**	**t**
1195	myrtenol	0.2	0.2	0.2	t	-	-	0.2	-
1204	verbenone	0.1	-	-	-	t	-	-	-
1207	trans-piperitol	-	-	-	-	-	-	0.4	-
1223	citronellol	t	-	-	-	-	-	0.2	-
1226	cis-carveol	-	-	-	-	-	-	0.3	-
1232	thymol, methyl ether	0.5	0.3	t	0.2	0.3 – 0.5	-	0.7	-
1249	piperitone	-	-	-	-	-	-	1.0	-
1253	trans-sabinyl acetate	0.2	-	-	-	-	-	-	-
1254	linalool acetate	0.2	0.2	0.3	0.2	-	-		-
1274	pregeijerene B	-	-	-	-	-	-	0.6	-
1284	**bornyl acetate**	**4.2**	**4.3**	**4.6**	**5.1**	**2.3 – 3.5**	**3.8**	**11.5**	**10.4**
1293	2-undecanone	0.3			0.1	t	-	0.2	-
1315	(2E,4E)-decadienal	0.1					-		-
1345	α-terpinyl acetate	1.8			1.4	0.8 – 1.0	-	2.4	t
1389	β-elemene	0.2	1.7	0.7	0.6	0.7 – 1.6	1.2	0.3	t
1417	**(E)-caryophyllene**	**5.3**	**5.3**	**4.5**	**5.0**	**4.8 – 6.0**	**5.3**	**5.9**	**5.1**
1439	aromadendrene	-	0.3	0.2	-	-	-	-	-
1442	6,9-guaiadiene	-	-	-	-	-	-	1.4	-
1454	α-humulene	0.8	0.6	0.5	0.8	0.8 – 1.0	-	1.2	1.0
1454	(E)-β-farnesene	0.2	-	-	-	-	-	0.1	-
1477	β-chamigrene	-	0.4	0.2	-	-	-	-	-
1478	γ-muurolene	0.2	0.4	0.2	-	t	-	-	0.2
1480	**germacrene D**	**1.7**	**9.9**	**4.0**	**5.6**	**3.9 -5.0**	**12.1**	**0.6**	**0.9**
1489	β-selinene	-	0.2	0.2	-	t	-	-	-

KI	compound	Bulgaria *15773* this study	Kosovo Oshlak Hajdari[1]	Kosovo Hajle Hajdari[1]	Sar Mtns. s. Kosovo Mitic[2]	Slovenia Julian Alps Bojovic[3]	Cambridge UK, Cult. Iannou[4]	*uncinata* central Italy Venditti[5]	*uncinata* cultivated Poland Celinski[6]
1500	bicyclogermacrene	1.1	2.2	3.4	1.7	1.4 – 2.7	-	0.3	1.1
1500	α-muurolene	0.3	0.6	0.3	0.6	t	-	0.3	-
1508	germacrene A	0.4	-	-	0.2	-	-		-
1513	γ-cadinene	0.6	0.7	0.6	0.8	0.3 – 0.8	-	0.3	0.4
1522	δ-cadinene	1.2	2.3	2.2	2.6	1.0 – 2.1	-	1.2	1.0
1537	α-cadinene	0.1	0.5	0.2	0.2	t	-	0.1	t
1561	(E)-nerolidol	0.1	0.6	0.7		-	-	0.1	-
1574	germacrene-D-4-ol	2.3	-	-	0.2	0.7 – 1.9	-		t
1577	spathulenol	-	1.1	1.3		-	-	0.8	-
1583	caryophyllene oxide	0.4	1.5	1.8	0.1	0.2 – 0.2	-	2.0	-
1608	humulene epoxide II	-	-	-	-	-	-	0.4	-
1638	epi-α-cadinol	0.4	0.7	0.7	-	-	-	1.3	-
1640	epi-α-muurolol	0.4	-	-	0.7	-	-	1.3	-
1644	α-muurolol	0.1	t	t	0.1	-	-	0.5	-
1652	**α-cadinol**	**1.1**	-	-	**0.9**	**0.4 – 0.8**	**2.1**	**4.1**	-
1710	pentadecanal	0.2	-	-	-	-	-	-	-
1880	(3Z)-hexenyl cinnama	0.5	-	-	-	-	-	-	-
1933	cyclohexadecanolide	t	-	-	-	-	-	-	-
1943	iso-cembrene	0.1	-	-	-	-	-	-	-
1987	manool oxide	0.2	0.2	0.2	0.1	t	-	1.0	-
2010	**13-epi-manool oxide**	**-**	-	-	-	**t**	-	**2.9**	-
2056	abietatriene	0.1	-	-	-	-	-	0.7	-
2087	abietadiene	0.1	t	t	-	-	-	0.5	-
2105	iso-abienol	0.2	-	-	-	-	-	-	-
2149	abienol	t	-	-	-	-	-	1.6	-
2153	abieta-(8(14,13j(15)-d	t	-	-	-	-	-	0.6	-
2243	**palustral (8,13-abietadien-18-al**	**1.0**	-	-	-	-	-	**8.7**	-
2274	**dehydro abietal**	**0.1**	-	-	-	-	-	**4.3**	-
2313	abietal	t	-	t	-	-	-	0.8	-

[1]Hajdari et al. 2015; [2]Mitic et al. 2017; [3]Bojovic et al. 2016; [4]Ioannou et al. 2014; [5]Venditti et al. 2013; [6]Celinski, et al. 2015.

Table 2. Variation in constituents of the leaf volatile oil of *P. mugo* subsp. *mugo* in a population in Bulgaria.

KI	compound	*15495*	*15496*	*15497*	*15498*	*15499*
921	tricyclene	0.2	0.7	0.2	0.2	0.3
924	α-thujene	0.1	t	0.7	0.1	0.3
932	**α-pinene**	**17.1**	**11.0**	**15.1**	**7.3**	**9.5**
946	camphene	1.0	2.5	1.1	1.0	1.2
969	sabinene	1.2	1.2	1.3	1.1	1.2
974	**β-pinene**	**3.3**	**3.0**	**3.1**	**1.7**	**3.0**
988	**myrcene**	**3.3**	**3.3**	**3.4**	**2.4**	**3.1**
1002	α-phellandrene	0.7	0.3	0.6	0.3	0.3
1008	**δ-3-carene**	**26.3**	**23.1**	**19.8**	**28.8**	**27.2**
1014	α-terpinene	0.2	0.2	0.3	0.2	0.2
1020	p-cymene	0.2	0.2	0.1	0.3	0.3
1024	limonene	t	t	t	t	t
1025	**β-phellandrene**	**12.8**	**13.8**	**21.6**	**16.6**	**18.8**
1044	(E)-β-ocimene	0.8	0.6	0.3	0.6	0.8
1054	γ-terpinene	0.3	0.4	0.4	0.4	0.4
1086	**terpinolene**	**3.6**	**3.4**	**4.6**	**3.1**	**3.9**
1118	cis-p-menth-2-en-1-ol	t	t	0.1	t	t
1132	cis-limonene oxide	t	t	t	t	t
1136	trans-sabinol	t	t	0.1	t	t
1165	borneol	t	0.3	0.1	0.3	0.3
1174	terpinen-4-ol	0.4	0.3	0.4	0.4	0.4
1176	m-cymen-8-ol	0.1	0.2	0.1	0.2	0.1
1179	p-cymen-8-ol	0.2	0.2	0.2	0.2	0.3
1186	α-terpineol	t	0.2	0.1	0.1	t
1195	myrtenol	t	t	t	t	t
1204	verbenone	t	t	t	0.2	t
1232	thymol, methyl ether	0.3	0.4	0.6	0.5	0.7
1253	trans-sabinyl acetate	t	0.1	0.2	0.2	t
1254	linalool acetate	t	0.2	t	t	t
1284	**bornyl acetate**	**1.6**	**8.9**	**2.8**	**3.0**	**4.0**
1293	2-undecanone	0.3	0.2	0.3	0.2	t
1315	(2E,4E)-decadienal	t	t	t	0.1	t
1345	α-terpinyl acetate	2.2	1.3	2.1	1.3	1.2
1389	β-elemene	0.2	0.2	0.2	0.3	0.1
1417	**(E)-caryophyllene**	**5.1**	**6.8**	**4.2**	**4.2**	**6.1**
1454	α-humulene	0.8	1.1	0.7	0.6	0.9
1454	(E)-β-farnesene	0.2	0.3	0.2	t	t
1478	γ-muurolene	0.2	0.1	0.2	0.2	t
1480	**germacrene D**	**2.9**	**0.4**	**1.3**	**1.5**	**2.5**
1500	bicyclogermacrene	1.3	0.7	0.7	2.1	0.8
1500	α-muurolene	0.3	0.2	0.3	0.3	0.3
1508	germacrene A	0.3	0.2	0.3	0.7	0.2
1513	γ-cadinene	0.6	0.3	0.4	1.0	0.4
1522	δ-cadinene	1.3	1.0	1.3	1.3	1.0
1537	α-cadinene	t	t	0.1	t	t
1561	(E)-nerolidol	t	0.1	0.2	t	t
1574	**germacrene-D-4-ol**	**2.4**	**1.3**	**2.4**	**3.3**	**1.2**
1583	caryophyllene oxide	0.3	0.3	0.2	0.9	0.4
1638	epi-α-cadinol	0.5	0.3	0.9	0.6	0.3
1640	epi-α-muurolol	0.4	0.3	0.2	0.5	0.3
1644	α-muurolol	t	0.1	0.2	0.2	t
1652	α-cadinol	1.2	0.8	1.2	1.6	0.9
1710	pentadecanal	0.1	0.4	0.1	t	0.3
1880	(3Z)-hexenyl cinnamate	0.5	0.7	0.4	0.1	t
1943	iso-cembrene	t	t	0.2	t	0.4
1987	manool oxide	0.2	0.2	0.2	0.2	0.4
2056	abietatriene	t	t	t	0.3	t
2087	abietadiene	t	t	0.1	0.1	t
2105	iso-abienol	t	0.5	0.2	t	t
2149	abienol	t	t	t	t	t
2153	abieta-(8(14,13j(15)-diene	t	t	t	t	t

KI	compound	*15495*	*15496*	*15497*	*15498*	*15499*
2243	**palustral (8,13-abietadien-18-al**	**0.6**	**1.2**	**0.6**	**1.5**	**0.7**
2274	dehydro abietal	t	0.1	t	0.3	t
2313	abietal	t	0.1	t	0.1	t

New and Noteworthy Epiphytic Ferns from the Urban Forests of Coastal Southern California, U.S.A.

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ABSTRACT

Davallia solida (G. Forst.) Sw. (Davalliaceae), *Phlebodium aureum* (L.) J. Sm. (Polypodiaceae), *Phlebodium pseudoaureum* (Cav.) Lellinger (Polypodiaceae), and *Rumohra adiantiformis* (G. Forst.) Ching (Dryopteridaceae) are epiphytic ferns native to the subtropical and tropical regions primarily of the Southern Hemisphere. These cultivated ornamental ferns are reported here for the first time growing without direct human assistance in the urban forests of coastal southern California. *Sphaeropteris cooperi* (Hook. ex Muell.) R.M. Tryon (Cyatheaceae), a popular ornamental tree fern native to the subtropical rainforests of eastern Australia, has naturalized on the rocky cliffs of Santa Catalina Island, southern California. We report the first documented mainland occurrences of this species growing as an epiphyte in coastal urban southern California. The known epiphytic occurrences documented for *Nephrolepis cordifolia* (L.) C. Presl (Nephrolepidaceae) and *Psilotum nudum* (L.) P. Beauv. (Psilotaceae) for the urban forests are also provided. Apparently for the first time in California or elsewhere, we report the known epiphytic occurrences of *Cyrtomium falcatum* (L.f.) C. Presl (Dryopteridaceae). Our findings suggest the southern California coastal urban forest environment promotes the dispersal and establishment of non-native epiphytes by facilitating interactions between cultivated phorophytes and several epiphytic ferns escaping cultivation. The ferns have been introduced by horticulture and escaped from gardens, events that represent an important colonization pathway for the invasion of southern California's urban forests. We hope that our findings will stimulate discourse and motivate others to study further the epiphytic ferns of California and other Mediterranean climate regions around the world.
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KEY WORDS: *Cyrtomium falcatum*, *Davallia solida*, *Nephrolepis cordifolia*, *Phlebodium aureum*, *Phlebodium pseudoaureum*, *Psilotum nudum*, *Rumohra adiantiformis*, *Sphaeropteris cooperi*, biological invasions, California, epiphytes, ornamental horticulture, *Phoenix canariensis*, urban forests.

The identity, frequency, and distribution of the non-native plants growing spontaneously outside of cultivation in California have historically been under-reported, even ignored (Hrusa et al. 2002). Also, the non-native plants are frequently given low priority by collectors, which has contributed to gaps in our knowledge (Baldwin et al. 2012). Because invasions of non-native species can significantly impact biodiversity, ecosystem function, human well-being, agriculture and/or commerce, scientists now pay more attention to new introductions (Mack & Lonsdale 2001; Pyšek et al. 2012; Vilà & Hulme 2017).

California has long been recognized as a hotspot for non-native plant invasions (Bossard et al. 2000). Accordingly, Dean et al. (2008) emphasized the importance of including introduced plants in regional floras, noting that specialized plant communities have also been invaded. Only recently, however, have the non-native epiphytes caught the attention of California botanists (Dean et al. 2008; Ritter 2011; Riefner & Smith 2015; Riefner 2016; CCH 2018) namely *Ficus carica* L., *F. microcarpa* L.f. and *F. rubiginosa* Desf. ex Vent. (Moraceae), *Nephrolepis cordifolia*, and *Psilotum nudum*.

Epiphytes account for approximately 9% of all vascular plant diversity in the world (Nieder et al. 2001; Zotz 2016). Our knowledge of epiphyte communities however, lags far behind that of terrestrial plants, and biogeographical studies are scarce (Kreft et al. 2004; Burns & Zotz 2010). Consequently, the vascular epiphyte diversity and composition for the temperate versus subtropical-tropical climate zones have been poorly documented (Zotz 2005; Hsu & Wolf 2009).

In many temperate regions epiphytes are understudied and little appreciated, but may not be devoid of interesting species (Zotz 2005). Brandes (2007), for example, reported an extensive list of the epiphytes colonizing *Phoenix canariensis* Chabaud (Arecaceae) cultivated in tourist centers around the Mediterranean Sea. Additionally, epiphytes are rarely identified in evaluations for the non-native plant invasions of the world's Mediterranean climate regions nor have they been considered as components contributing to global biotic homogenization (Arianoutsou et al. 2013). Accordingly, little work has been done to document the non-native epiphytic ferns growing spontaneously in coastal southern California.

In this paper, we document occurrences of eight non-native ferns growing epiphytically outside of cultivation in the urban forests of coastal southern California. These ferns grow primarily on the trunks of *Phoenix canariensis*, which is cultivated widely along streets and in parks. Figure 1 depicts a human-planted urban forest in the Mediterranean climate of coastal southern California; it also depicts *P. canariensis* in typical urban settings colonized by the epiphytic ferns.

MATERIALS AND METHODS

We reviewed various classification schemes for the vascular epiphytes and here employ the definition prepared by Zotz (2016). In addition, we describe the urban forest, and outline the field survey program implemented to develop a preliminary conspectus of the non-native epiphytic ferns growing spontaneously in the urban forests of coastal southern California.

Defining Vascular Epiphytes

Vascular epiphytes are "plants that germinate and root non-parasitically on other plants at all stages of life" (Zotz 2016). Many epiphytes, however, grow occasionally on rocks or soil, and conversely many primarily terrestrial plants grow occasionally on living substrates (Zotz 2016). Therefore, this definition resolves most previous problematic classifications for plants that exhibit variable degrees of fidelity to epiphytic life (Zotz 2016).

Zotz (2016) consolidated previous classification schemes into a format that can be readily applied worldwide: 'obligate' epiphytes are species with >95% occurrence of individuals in a particular region growing epiphytically; 'accidental' epiphytes include species with >95% occurrence of individuals growing terrestrially in a region; and 'facultative' epiphytes are those species demonstrating a frequency of occurrence between these two categories, i.e., individuals that are terrestrial and epiphytic. Obligate epiphytes are referable to 'true, typical or holoepiphyte' classifications (Zotz 2016). Therefore, by utilizing a common definition for epiphytic classification scientists can more accurately characterize the structure of epiphytic communities and their biogeographical patterns (Zotz & Bader 2011).

The Urban Forest

An urban area, in a broad sense and adopted here, is defined as a human settlement with high population density and infrastructure of built environment (Wikipedia 2018a). An urban forest is a network comprising all woodlands, groups of trees and individual trees located in urban and peri-urban (transition zone between urban and rural) environments (Salbitano et al. 2016).

America's urban forests comprise over 130 million acres of parks, street trees, landscaped boulevards, gardens, greenways, river, wetland, and coastal promenades, nature preserves, and trees growing at former industrial sites (USDA, USFS 2018). In California, there are an estimated 9.1 million street trees, which comprise about 10–20% of the total urban forest (McPherson et al. 2015). Urban forests provide habitats and services that enrich the quality of life for humans (Jim & Chen 2009). They also provide resources for animals and plants, conserve native biodiversity, and sustain ecological functions in landscapes that are increasingly fragmented by development (Howenstine 1993; Pyšek 1998; Nowak et al. 2010; Rega et al. 2015). Urban environments, however, can be a source of invasive non-native ornamental plants that escape human-planted landscapes (Dehnen-Schmutz et al. 2007).

In southern California, urban forests are diverse, human-planted ecosystems comprising tree species imported from diverse habitats, climates, and continents (Pataki et al. 2013; Avolio et al. 2105). Lesser (1996) reported 257 species in 123 genera for 21 southern California cities. Further, Jenerette et al. (2016) studied the composition of urban forests in 20 cities within the United States and Canada; topping the list for diversity are Los Angeles with 104 tree species, Santa Barbara 85, Irvine 77, and San Francisco with 77. Thus, the urban forests of southern California have been acclaimed as a "Garden of Eden" (Pincetl et al. 2013).

Southern California is also renowned for its iconic and ubiquitous cultivation of palm trees (Farmer 2013; Pataki et al. 2013). Palms are planted frequently along streets and boulevards, parks, business plazas, and in residential communities (Hatch 2007; Avolio et al. 2015); noteworthy are *Washingtonia robusta* H. Wendl., *Washingtonia filifera* (Lindl.) H. Wendl., *Syagrus romanzoffiana* (Cham.) Glassman, *Phoenix canariensis*, *Phoenix dactylifera* L., and *Butia capitata* (Mart.) Becc.

Study Area: Focused Field Survey Program

A survey program to document epiphytic ferns was implemented following the discovery of *Nephrolepis cordifolia* growing on palms in coastal urban southern California (Riefner & Smith 2015). Riefner surveyed coastal Los Angeles, Orange, and San Diego counties from 2014–2018 to document the epiphytic ferns growing spontaneously outside of cultivation in coastal southern California. The surveys focused on public landscapes along streets, boulevards, and freeways, greenbelts around residential housing tracks or business plazas, and county parks and recreation areas.

Wherever epiphytic ferns were located, including palm or non-palm tree hosts, data collection included a physical description of the location, GPS coordinates, notes on the species and population size, host tree species, and photographic documentation. Herbarium specimens were collected, with the exception of sites where ferns were observed growing beyond reach; these ferns were photographed for documentation in lieu of collecting specimens, and the photographs will be posted online.

Scientific names follow the phylogenetic system adopted by the Pteridophyte Phylogeny Group (PPG I 2016). The PPG provides a comprehensive consensus classification by employing a community-based approach following the main tenets of Smith et al. (2006).

RESULTS

No native California ferns were observed. Rather, we documented eight non-native epiphytic fern species in six families originating from a diversity of geographic regions. Table 1 lists the species, nativity, preliminary epiphytic classification, and the host tree(s) recorded during this study.

Table 1. Non-Native Epiphytic Ferns Recorded During the Study for Los Angeles, Orange, and San Diego Counties, Coastal Southern California.

Species	Family	Nativity	Epiphytic Class	Phorophyte(s)
New to California				
Davallia solida	Davalliaceae	Australasia	Obligate	*Phoenix canariensis*
Phlebodium aureum	Polypodiaceae	Subtropical-Tropical Americas	Obligate	*Phoenix canariensis*
Phlebodium pseudoaureum	Polypodiaceae	Subtropical-Tropical Americas	Obligate	*Phoenix canariensis*
Rumohra adiantiformis	Dryopteridaceae	Southern Hemisphere	Obligate	*Phoenix canariensis*, *Butia capitata*
New for the Mainland Coast				
Sphaeropteris cooperi	Cyatheaceae	Eastern Australia	Facultative	*Phoenix canariensis*, *Butia capitata*
Noteworthy Species				
Nephrolepis cordifolia	Nephrolepidaceae	Australasia-Pacific Islands, Neotropics	Facultative	*Phoenix canariensis*, *Butia capitata*, *Washingtonia robusta*, *Pinus pinea*
Psilotum nudum	Psilotaceae	Cosmopolitan: Warm Temperate to Tropical	Facultative	*Syagrus romanzoffiana*, *Phoenix canariensis*
First Documentation of Epiphytic Behavior				
Cyrtomium falcatum	Dryopteridaceae	East Asia	Accidental	*Phoenix canariensis*

***Davallia solida*, *Phlebodium aureum*, *Phlebodium pseudoaureum*, and *Rumohra adiantiformis* new to California**

Davallia solida, *Phlebodium aureum*, *P. pseudoaureum*, and *Rumohra adiantiformis* have not been previously cited growing spontaneously outside of cultivation in California, including Nauman (1993a), Hrusa et al. (2002), Roberts et al. (2004), Clarke et al. (2007), DiTomaso and Healy (2007), Dean et al. (2008), Roberts (2008), Prigge and Gibson (2012), Smith (2012a,b), Rebman and Simpson (2014), Jepson Flora Project (2018), and the USDA, NRCS (2018). No voucher specimens for *D. solida*, *P. aureum*, *P. pseudoaureum*, or *R. adiantiformis* growing outside of cultivation have been posted for California by the Consortium of California Herbaria (CCH 2018) or the Southwest Environmental Information Network (SEINet 2018).

In addition, *Phlebodium aureum*, *P. pseudoaureum*, and *Rumohra adiantiformis* have not been documented previously growing outside of cultivation along the Pacific Coast of North America. In the United States and its territories, *P. aureum* is native to Florida, Puerto Rico, and the Virgin Islands, and has naturalized in Hawai'i (Nelson 2000; Vernon & Ranker 2013; Randall 2017; USDA, NRCS 2018). However, there is considerable disagreement regarding its native versus introduced range for Alabama, Georgia, and South Carolina (Snyder & Bruce 1986; Weakley 2015; USDA, NRCS 2018). *Phlebodium pseudoaureum* is native to Puerto Rico, but has not naturalized in Hawai'i (Lellinger 1985; Imada 2012; Vernon & Ranker 2013; USDA, NRCS 2018). There is also considerable disagreement regarding its nativity or occurrence in Florida (Proctor 1985, 1989; Nauman 1993a; Weakley 2015; USDA, NRCS 2018). *Rumohra adiantiformis* is native to Puerto Rico and has naturalized in Florida, but is not known to occur outside of cultivation elsewhere in the flora region (Lellinger 1985; Vernon & Ranker 2013; Randall 2017; USDA, NRCS 2018).

Voucher specimens (duplicates to be distributed), all from **U.S.A.: CALIFORNIA: *Davallia solida*: Orange Co.:** City of San Clemente, coastal bluff W of West Vista Blanca and S of San Clemente State Beach, 33.39906N -117.60136W, elev. ca. 21 m, rare, epiphytic on trunk of *Phoenix canariensis*, growing with *Asparagus* sp. and *Nephrolepis cordifolia*, 9 Sep 2018, *Riefner 18-115* (UC). **San Diego Co.:** City of Oceanside, along South Freeman St. at Missouri Ave., 33.19235N -117.37518W, elev. ca. 23 m, large clump of rhizomatous plants epiphytic on the trunk of a roadside *Phoenix canariensis*, growing with *Nephrolepis cordifolia*, 30 Sep 2016, *Riefner 16-385* (UC). ***Phlebodium aureum*: Los Angeles Co.:** City of Malibu, Eastern Malibu, W of Carbon Canyon Rd., N side Pacific Coast Highway at Carbon Mesa Rd., 34.03905N -118.65394W, elev. ca. 31 m, epiphytic near ground level to ca. 3 m off the ground, well-established on the trunk of *Phoenix canariensis*, urban landscape, rhizomes intertwined within dense growth of *Ficus rubiginosa* and *Nephrolepis cordifolia*, irrigation spray drift present, 13 Dec 2015, *Riefner 15-469* (UC). ***Phlebodium pseudoaureum*: Orange Co.:** City of Laguna Beach, Aliso Beach Park, W of Coast Highway, 33.50984N -117.75135W, elev. ca. 4 m, rare, epiphytic on trunk of *Phoenix canariensis* cultivated along roadside in urban greenbelt, growing with *Cyrtomium falcatum*, 18 Aug 2018, *Riefner 18-110* (UC). ***Rumohra adiantiformis*: Los Angeles Co.:** City of Venice, along Venice Blvd. between Lyceum Ave. and Walgrove Ave., 33.99804N -118.443365W, elev. ca. 11 m, rare epiphyte on the trunk of *Phoenix canariensis,* urban landscape, irrigation spray present, growing with *Cyrtomium falcatum, Nephrolepis cordifolia*, *Sphaeropteris cooperi,* and *Ficus carica*, 24 Sep 2016, *Riefner 16-375* (UC); City of Long Beach, Belmont Park, Alamitos Bay, Bay Shore Ave. along Appian Way, 33.76007N -118.123531W, elev. ca. 3 m, local, epiphytic on trunk of cultivated *Butia capitata*, growing with *Nephrolepis cordifolia*, urban landscape, 10 Jun 2018, *Riefner 18-64* (UC). **Orange Co.:** City of San Clemente, Cyprus Shore, coastal bluff W of Calle Ariana and S of San Clemente State Beach, 33.39602N -117.59955W, elev. ca. 12 m, abundant epiphytic on trunk of *Phoenix canariensis* cultivated in greenbelt, irrigation spray drift present, growing with *Asparagus* sp., 9 Sep 2018, *Riefner 18-118* (UC). **San Diego Co.:** City of Carlsbad, N of Batiquitos Lagoon, along Batiquitos Dr. at Gabbiano Ln., 33.09822N -117.30276W, elev. ca. 26 m, epiphyte on the trunk of *Phoenix canariensis,* urban landscape, irrigation spray present, 1 Feb 2017, *Riefner 17-52* (UC); City of

Carlsbad, N side of Agua Hedionda Lagoon, along Adams St. E of Highland Dr., 33.14519N -117.32581W, elev. ca. 15 m, locally abundant, epiphyte on trunks of cultivated *Phoenix canariensis* street trees, growing with *Nephrolepis cordifolia*, 30 Jun 2017, *Riefner 17-322* (UC).

Davallia solida **(Davalliaceae):** *Davallia sensu lato* is widely distributed from the Atlantic Islands to Africa, southern Asia to Malaysia, Polynesia, Australia, New Zealand, and the Pacific Islands (Nooteboom 1994; Yuyen & Boonkerd 2002; Xing et al. 2013). *Davallia* species are small to medium-sized ferns that in the wild are usually epiphytic. Most species are easily recognized by their long-creeping and densely scaly rhizomes, short phyllopodia, finely dissected or pinnatifid blades, and cup-shaped indusia and sori that are nearly marginal on pinnae (Hoshizaki & Moran 2001). Many of the thin-textured davallias are drought-sensitive (Hoshizaki & Moran 2001). *Davallia solida*, however, is drought-tolerant and sheds fronds or parts of the blade during dry seasons (Yuyen & Boonkerd 2002).

A leathery evergreen perennial, *D. solida* is an epiphyte, but occasionally may be epilithic (growing on rocks) or terrestrial. It is widespread in China, Cambodia, Thailand, India, Indonesia, Malaysia, Myanmar, Australia, Papua New Guinea, Philippines, Sri Lanka, Thailand, Vietnam, and the Pacific Islands (Xing et al. 2013; ABRS 2018). Generally, *D. solida* is easily recognized by its dark rhizome, rhizome scales that are ovate-lanceolate with a long subulate apex and ciliate margins, leathery lamina, and tubular indusia (Nooteboom 1994; Xing et al. 2013).

Davallia solida grows in exposed places or in deep shade, including open rocky habitats and savannas or in rainforests from sea level to about 1,500 m (Nooteboom 1994; Xing et al. 2013). In some regions, *D. solida* has been identified as a microhabitat specialist, growing almost exclusively on mangroves (Nakamura 2000; Hayasaka et al. 2012). *Davallia solida* also grows in human-modified habitats, including rubber agro-forest plantations, urban green spaces in Indonesia, and an urban nature preserve in Singapore (Wee 1995; Beukema et al. 2007; Ulfa et al. 2013).

Widely cultivated as an ornamental (Hoshizaki & Moran 2001), including in Hawaiʻi (Imada et al. 2018), *D. solida* apparently is not commonly available in the California retail market (Perry 2010; Brenzel 2012). *Davallia solida* is also grown in Florida, Costa Rica, and Africa for commercial production of cut-fronds used by florists (McConnell et al. 1989; Stamps 2017).

Davallia solida is not known to escape cultivation in the United States and its territories (Randall 2017; USDA, NRCS 2018), including Hawaiʻi (Imada 2012; Vernon & Ranker 2013), nor has it escaped in Europe (Euro+Med PlantBase 2018). The two known locations documented for *D. solida* growing outside of cultivation in southern California are depicted in Figure 2. Its epiphytic habitat on *Phoenix canariensis* and morphological features are shown in Figure 3, Photographs A–C.

Phlebodium aureum **(Polypodiaceae):** *Phlebodium* (R. Br.) J. Sm. comprises about four species of primarily epiphytic, or less frequently epilithic ferns, that occur in subtropical and tropical environments of the New World (Mickel & Smith 2004). *Phlebodium aureum* (syn., *Polypodium aureum* L.), a fertile tetraploid, is believed to have arisen through allopolyploidy following hybridization between *P. decumanum* (Willd.) J. Sm. and *P. pseudoaureum* (Cav.) Lellinger (Nauman 1993a; Mickel & Smith 2004). *Phlebodium aureum* is similar to *P. pseudoaureum*, but differs in having sori arranged usually in two rows between the costa and pinna margin, and its fronds are more deeply lobed with generally broader segments (Hoshizaki & Moran 2001; Mickel & Smith 2004). Because they are easily confused, Mickel and Smith (2004) reported that collections identified as *P. aureum* in Mexico are *P. pseudoaureum* (Cav.) Lellinger (syn., *P. areolatum* [Humb. & Bonpl. ex Willd.] J. Sm.).

Phlebodium aureum, a deciduous medium to large perennial, is epiphytic on a variety of trees, decaying logs or humus. In the southeastern United States, Lellinger (1985) and Nauman (1993a) noted it

grows frequently among old leaf bases on the trunks of *Sabal palmetto* (Walt.) Lodd. It is also epiphytic in crotches and crevices of other trees, particularly *Quercus virginiana* Mill. (Fagaceae), but rarely is terrestrial growing on calcareous soils, or epilithic on limestone and masonry (Weakley 2015; Wunderlin et al. 2018). Thereby, *P. aureum* occupies a variety of habitats and microclimates, including mesic to hydric hammocks, swamps, coastal strand and beaches, pine flatwoods, ditches, and ruderal sites (Nauman 1993a; Wunderlin et al. 2018).

Phlebodium aureum can also grow in dry sunny locations, but then the fronds are deciduous and seasonally shed during drought (Benzing 1990; Crouch et al. 2011). Phyllopodia (basal portions of petioles, proximal to articulation planes that remain attached to the rhizomes after old fronds abscise) are an adaptation to an epiphytic habitat and seasonal growing conditions. Deciduous fronds expose the phyllopodia that may help with identification of *Phlebodium* species during drought.

Phlebodium aureum is reported as indigenous to Alabama, Florida, Georgia, Mexico, the West Indies, and Central and South America (Nauman 1993a; USDA, NRCS 2018). However, there is considerable disagreement regarding its range and nativity in the southeastern United States (Lellinger 1985; Weakley 2015; Keener et al. 2018). Weakley (2015) also reported the species has been introduced to Beaufort, Jasper, and Charleston counties, South Carolina, via planting of palmettos.

Because *P. aureum* is epiphytic, epilithic or terrestrial, and tolerant of harsh conditions, it has naturalized in many warm-temperate, subtropical, and tropical regions (Randal 2017). *Phlebodium aureum* is a well-known weed in Hawaiʻi. It was first documented in 1909 (Wagner 1950; Wilson 1996) and now grows on all of the main islands (Imada 2012). *Phlebodium aureum* is epiphytic in forests and near habitations, but rarely grows on buildings in Hawaiʻi (Wilson 1996). Wagner (1950) observed it as a volunteer growing with *Nephrolepis* sp. on *Phoenix canariensis* in a yard near the Bishop Museum.

Phlebodium aureum is commonly cultivated in South Africa and has escaped in the vicinity of Durban, KwaZulu-Natal, and at Port St. Johns in the Eastern Cape (Crouch et al. 2011). *Phlebodium aureum* grows as an epiphyte in brightly lit situations in moist evergreen riverine forests and their margins, occasionally in exposed situations on ledges of buildings, but is found rarely naturalized on soil (Crouch et al. 2011). *Phlebodium aureum* has also naturalized on Mauritius (Lorence 1978) and sparingly in New South Wales and Queensland, Australia (Pellow et al. 2009; ABRS 2018). It is an occasional escape in the Mediterranean region, i.e., Madeira (Euro+Med PlantBase 2018).

Outside of cultivation in southern California, *P. aureum* grows among the leaf bases and crotches on the trunk of *Phoenix canariensis* within the drift spray zone of landscape irrigation facilities. It is closely associated with other epiphytic ferns and the hemiepiphytic species of *Ficus*. The single known location documented for *P. aureum* is depicted in Figure 2. Its epiphytic habitat on *P. canariensis* and distinctive morphological features are depicted in Figure 4, Photographs A–C.

***Phlebodium pseudoaureum* (Polypodiaceae):** *Phlebodium pseudoaureum* is a deciduous medium to large perennial that is epiphytic, epilithic, or terrestrial in numerous tropical forest ecosystems (Mickel & Smith 2004). Widespread in Central and South America, *P. pseudoaureum* is also reported to be native for Florida and Puerto Rico, but not Hawaiʻi (Proctor 1989; Davidse et al. 1995; Mickel & Smith 2004; Imada 2012; USDA, NRCS 2018). Because it is similar to and can be confused with *P. aureum* (Nauman 1993a; Mickel & Smith 2004), its occurrence has been disputed for Florida (Lellinger 1985; Proctor 1985, 1989; Nauman 1993a; Weakley 2015; Wunderlin et al. 2018). *Phlebodium pseudoaureum* differs in having sori arranged in a single row between the costa and pinna margin, venation, and stiffer, slightly more leathery blades (Hoshizaki & Moran 2001; Mickel & Smith 2004).

Phlebodium pseudoaureum is not known to escape cultivation in the United States (Randall 2017; USDA, NRCS 2018), including in Hawai'i (Vernon & Ranker 2013). Also, it has not naturalized in Europe (Euro+Med PlantBase 2018), Australia (ABRS 2018), New Zealand (NZPCN 2018), or South Africa (Crouch et al. 2011); however, wherever *P. aureum* has naturalized herbarium specimens should be reexamined and additional collections may be needed to assess whether *P. pseudoaureum* has been overlooked.

The epiphytic habitat poses novel ecophysiological challenges to growth and survival, which are relevant for ferns that alternate their life cycle between two distinct free-living phases, gametophytes and sporophytes (Watkins & Cardelús 2012). Since epiphytes are often absent in dry habitats, water shortage and tolerance to dehydration is thought to be the primary limiting abiotic factors for establishment and survival (Benzing 1990; Zotz 2016). True desiccation tolerance in the fern sporophyte phase is known, but likely exists in relatively few species, such as the cheilanthoid species that are frequently associated with xeric habitats (Proctor & Pence 2002; Watkins et al. 2007). Watkins et al. (2007), however, demonstrated that desiccation-tolerance in the gametophytic phase may explain how some ferns have successfully colonized drought-prone habitats, including gametophytes of tropical species such as *P. pseudoaureum*. Gametophytes vary considerably in water content throughout the day, or season, and must be able to withstand long periods of desiccation, especially epiphytic taxa (Watkins 2006). In addition to shedding fronds during drought, *P. pseudoaureum* exhibits other physiological characteristics that may explain how it can colonize an epiphytic habitat in the California arid Mediterranean climate.

Outside of cultivation in southern California, *P. pseudoaureum* grows among the leaf bases and feeder roots on *Phoenix canariensis*, within the drift spray zone of landscape irrigation facilities. The single location documented for *P. pseudoaureum* is depicted in Figure 2. Its epiphytic habitat on *P. canariensis* and distinctive morphological features are shown in Figure 4, Photographs D–F.

***Rumohra adiantiformis* (Dryopteridaceae):** *Rumohra* species are medium-sized mostly epiphytic or terrestrial ferns that sometimes are considered to be related to *Polystichum* because of their peltate indusia (Hoshizaki & Moran 2001). *Rumohra* species, however, are generally easily recognized by their long-creeping, densely scaly rhizomes with fronds borne in two rows, deltate usually thick-textured decompound blades, and non-spinulose segment margins (Hoshizaki & Moran 2001).

Rumohra adiantiformis, a leathery evergreen perennial, is believed to be indigenous to South America (e.g., Argentina, Bolivia, Brazil, Chile, Peru, Uruguay, and Venezuela), the Greater Antilles, South Africa, Madagascar, Mauritius in the Indian Ocean, Australia, Papua New Guinea, New Zealand, and Tasmania (Lorence 1978; Proctor 1989; Mickel & Smith 2004; ABRS 2018; Kessler et al. 2018). It was not documented for Mesoamerica by Davidse et al. (1995), and the single collection from Jalisco, Mexico, may represent an escape from cultivation (Mickel & Smith 2004).

Rumohra adiantiformis grows in moist to wet forests commonly as a vigorous epiphyte, often on rotting trunks, tree ferns, and a variety of host trees, on rocks, and occasionally as a terrestrial plant (Duncan 1994; ABRS 2018). In Jamaica, Proctor (1985) reported it on trees and rotten logs, rarely in humus-filled pockets of limestone cliffs, widespread in forested regions but mostly rare or uncommon. It can tolerate sandy or loam soils of either poor or good fertility, long periods of dryness, and cool conditions, including frosts (Garrett 1996). Owing, in part, to its coriaceous fronds, *R. adiantiformis* is able to resist desiccation (Lorence 1978).

Cultivated as an ornamental groundcover or grown in pots, *R. adiantiformis* is also prized by florists for its long-lasting cut-fronds used for floral arrangements (Hoshizaki & Moran 2001; De Souza et al. 2006). It is one of the most economically important ferns and is propagated widely for the horticultural industry (Thomas 1999). Both wild and cultivated *R. adiantiformis* are highly variable, but

the peltate indusia become black when mature, robust long-creeping rhizomes, and the leathery leaves allow for easy identification (Hoshizaki & Moran 2001).

Rumohra adiantiformis has naturalized in subtropical and Mediterranean regions, including Florida in the United States and Europe (Verloove 2006; Randal 2017; Wunderlin et al. 2018). USDA, NRCS (2018) reported *R. adiantiformis* is native to Florida. However, others consider these occurrences to be introductions (Lellinger 1985; Wunderlin et al. 2018) as they often grow in suburban woodlands, disturbed areas, and along roadsides (Weakley 2015). *Rumohra adiantiformis*, however, has not naturalized in the Hawaiian Islands (Imada 2012; Vernon & Ranker 2013; USDA, NRCS 2018).

The known locations documented for *R. adiantiformis* growing outside of cultivation in southern California are depicted in Figure 2. It is epiphytic on *Phoenix canariensis*, rarely on *Butia capitata*, entirely within the influence of irrigation facilities. Its epiphytic habitat on *P. canariensis* and distinctive indusia are depicted in Figure 5, Photographs A–B.

***Sphaeropteris cooperi* (Cyatheaceae) new to the California mainland coast:** *Sphaeropteris* Bernh. is native to the American tropics, India, southeastern Asia, Australia, New Zealand, and the Pacific Islands (Hoshizaki & Moran 2001). *Sphaeropteris cooperi* (syn., *Cyathea cooperi* [Hook. ex F. Muell.] Domin) is a medium to large, fast growing tree fern (to 12 m tall) that is native to the subtropical rainforests of eastern Australia (ABRS 2018). It grows along forest margins, steamsides, gullies, and many open habitats, mostly in the coastal lowlands (McCarthy 1998).

Because of its fast growth rate, hardiness, and aesthetic appeal, *S. cooperi* is the most frequently cultivated tree fern in many temperate, subtropical, and tropical regions around the world, including Hawaiʻi, California, and elsewhere (Hoshizaki & Moran 2001; Brenzel 2012; Imada et al. 2018). *Sphaeropteris cooperi* is widely known for its adaptability to a variety of soil types, substrates, vegetation communities, tolerance to cold weather or extreme heat, with elevations ranging from sea level to nearly 1,400 m (Large & Braggins 2004; Hoshizaki & Moran 2001; Robinson et al. 2010). In the Hawaiian rainforests, *S. cooperi* is primarily terrestrial (77%), but 20% of the populations studied also grow on fallen logs, and 3% of the occurrences grow as epiphytes (Medeiros et al. 1992).

Sphaeropteris cooperi is known to escape cultivation and has naturalized in temperate, subtropical, and tropical regions around the world, including California, Florida, Oregon, and Hawaiʻi in the United States, the Azores and Madeira in Europe, New Zealand, southwest Australia (outside its native range), South Africa, Mauritius, and numerous Pacific Islands (Lorence 1978; Medeiros et al. 1992; Wood 2008; Rosatti 2013; Baard & Kraaij 2014; Randall 2017; ABRS 2018; Euro+Med PlantBase 2018; NZPCN 2018; USDA, NRCS 2018). In Hawaiʻi, *Sphaeropteris cooperi* is a serious threat to native ecosystems (Simberloff & Rejmánek 2011). This species aggressively invades natural areas, alters habitats, and out-competes native species, including native tree ferns, *Cibotium* spp. (Medeiros et al. 1992; Durand & Goldstein 2001; Palmer 2003). Displacement of the native tree ferns also alters the composition of epiphyte communities (Medeiros et al. 1993). *Sphaeropteris cooperi* is also invasive in South Africa (Baard & Kraaij 2014).

On the west coast of the United States, *S. cooperi* was first reported outside of cultivation in coastal southern Oregon (Wood 2008), and subsequently on Santa Catalina Island, southern California (Clark & Summers 2013). On Santa Catalina Island, *S. cooperi* grows on exposed granitic and sandstone cliffs in microhabitats that accumulate water, but also on dry, barren rock faces seemingly unsuitable for a species native to wet climates (Clark & Summers 2013). Accordingly, the dry microhabitat seen on Santa Catalina Island and the summer-dry Mediterranean climate in southern California represent conditions generally believed to be inhospitable for naturalization of a tree fern native to subtropical environments

(Durand & Goldstein 2001; Clark & Summers 2013). *Sphaeropteris cooperi* can survive a wide range of conditions, especially with adequate water, so it is expected to spread further in California (Rosatti 2013).

Many epiphytic populations for *S. cooperi* documented outside of cultivation on the California mainland grow on upper palm tree trunks that are not associated with direct irrigation waters. Because of recurrent severe drought, some epiphytic populations may not persist, especially following trimming of palm fronds that exposes plants to the desiccating effects of direct sunlight. *Sphaeropteris cooperi* is epiphytic primarily on *Phoenix canariensis*, rarely on *Butia capitata*. The known epiphytic locations of *S. cooperi* documented for southern California are depicted in Figure 2. Its epiphytic habitat on the upper trunks of *P. canariensis* is depicted in Figure 5, Photographs C–D.

Representative voucher specimens (duplicates to be distributed; data for many additional collections available at Consortium of California Herbaria)—**U.S.A.: CALIFORNIA: Los Angeles Co.:** City of Santa Monica, Pacific Palisades, Bay Club Dr. at Pacific Coast Hwy., 34.04187N -118.54614, elev. ca. 16 m, locally common, epiphyte on the trunk of *Phoenix canariensis*, urban landscape, irrigation spray drift present, 25 Nov 2017, *Riefner 17-543* (UC); City of Malibu, along Pacific Coast Hwy. at Ramirez Mesa Dr., uncommon epiphyte, upper trunk of *Phoenix canariensis*, plants under drought stress, urban landscape, 34.02254N -118.79080W, elev. ca. 30 m, 25 Nov 2017, *Riefner 17-546* (photographic documentation). **Orange Co.:** City of Dana Point, adventive in retail nursery, Pacific Coast Hwy., Ruby Lantern and San Mareno Place, 33.46628N -117.705127W, elev. ca. 49 m, under ledge of concrete block wall, not intentionally planted, irrigation runoff present, growing with *Soleirolia soleirolii*, 17 Feb 2016, *Riefner 16-50* (UC); City of Laguna Beach, Ruby Street Park, 33.52614N -117.76953W, elev. ca. 16 m, rare epiphyte on upper trunk of cultivated *Phoenix canariensis*, 4 Sep 2018, *Riefner 18-113* (photographic documentation); City of Laguna Beach, Glenneyre St. near Center St., 33.52896N -117.76996W, elev. ca. 40 m, two plants epiphytic on upper trunk of cultivated *Phoenix canariensis*, 15 Sep 2018, *Riefner 18-120* (photographic documentation). **San Diego Co.:** City of Carlsbad, along Highland Dr. at Carlsbad Village Dr., 33.16587N -117.33770W, elev. ca. 48 m, business plaza landscape, epiphytic on upper trunk of *Phoenix canariensis* with *Nephrolepis cordifolia*, beyond influence of urban irrigation waters, 20 Nov 2015, *Riefner 15-447* (photographic documentation); City of Carlsbad, N of Batiquitos Lagoon, end of Merlo Ct. and Piovana Ct. off Gabbiano Ln., 33.09621N -117.30136N, elev. ca. 22 m, epiphyte mostly on upper trunks of *Phoenix canariensis*, cultivated street trees, 11 Nov 2017, *Riefner 17-524* (UC).

Noteworthy Epiphytic Species: *Nephrolepis cordifolia* and *Psilotum nudum* have been reported previously from California (Riefner & Smith 2015; Farrar 2018). However, neither species has been reported from California by the USDA, NRCS (2018).

Nephrolepis cordifolia **(Nephrolepidaceae):** *Nephrolepis cordifolia* is terrestrial, epiphytic, or epilithic. It grows in moist to wet shady places, rain forests, coastal shrublands, wetland and riparian habitats, on epiphyte perches and particularly on palm trunks, limestone ledges and other cliff and rock outcrop habitats, urban areas, old home sites, roadsides, or waste places in Florida, Mexico, West Indies, Central America, South America, Africa, Europe, Southeast Asia, the Pacific Islands and Hawaiʻi, Australia, New Zealand, and elsewhere (Nauman 1993b; Hovenkamp & Miyamoto 2005; Randall 2017; ABRS 2018; NZPCN 2018; Euro+Med PlantBase 2018; USDA, NRCS 2018).

In southern California, outside of cultivation *N. cordifolia* has been documented from riparian habitats and on coastal cliff and rock outcrops in native plant communities, but it occurs more frequently in urban environments (Riefner & Smith 2015; CCH 2018).

Representative voucher specimens (duplicates to be distributed; data for many additional collections available at Consortium of California Herbaria)—**U.S.A.: CALIFORNIA: Los Angeles Co.:** City of Los Angeles, E of Westwood Blvd. and W Overland Ave., N side of I-10 Freeway, 34.03206N -

118.41783W, elev. ca. 70 m, epiphyte on trunk of *Phoenix canariensis* cultivated on freeway landscape, 5 Sep 2016, *Riefner 16-364* (UC); City of Malibu, along Pacific Coast Hwy. at Ramirez Mesa Dr., uncommon epiphyte on the upper trunk of *Phoenix canariensis*, growing with *Sphaeropteris cooperi* beyond influence of irrigation sprays, urban landscape, 34.02254N -118.79080W, elev. ca. 30 m, 25 Nov 2017, *Riefner 17-547* (photographic documentation). **Orange Co.:** City of Laguna Beach, W side of El Toro Rd., N of Canyon Hill Dr., 33.59639N -117.74883W, elev. ca. 112 m, uncommon, epiphyte on trunk of *Phoenix canariensis*, within irrigation spray zone, urban landscape, 3 Nov 2017, *Riefner 17-511* (UC); City of Laguna Beach, Glenneyre St. near Diamond St., 33.52837N -117.76919W, elev. ca. 40 m, abundant, epiphytic on upper trunk of cultivated *Phoenix canariensis*, growing with *Asparagus* sp., beyond influence of irrigation waters, 15 Sep 2018, *Riefner 18-121* (photographic documentation). **San Diego Co.:** City of Carlsbad, Highland Dr. at Carlsbad Village Dr., 33.16587N -117.33770W, elev. ca. 48 m, business plaza landscape, epiphytic on upper trunk of *Phoenix canariensis* with *Sphaeropteris cooperi*, beyond influence of urban irrigation waters, 20 Nov 2015, *Riefner 15-448* (photographic documentation); City of San Diego, Mission Valley, Hotel Circle South, vicinity of Taylor St., 32.75910N -117.181768W, elev. ca. 12 m, epiphytic on upper trunk of *Phoenix canariensis* beyond influence of urban irrigation waters, business plaza landscape, 14 Jan 2017, *Riefner 17-22* (photographic documentation); City of San Diego, Point Loma, along Catalina Blvd. near Pio Pico St., 32.71473N -117.24773W, elev. ca. 97 m, epiphytic on upper trunk of cultivated *Phoenix canariensis*, beyond influence of urban irrigation waters, roadside landscape, 16 Jun 2018, *Riefner 18-74* (photographic documentation).

The known epiphytic occurrences for *N. cordifolia* documented outside of cultivation are depicted in Figure 6, including the populations reported previously by Riefner and Smith (2015) and CCH (2018). Its preferred host tree is *Phoenix canariensis*, but *N. cordifolia* is also epiphytic on *Butia capitata*, *Washingtonia robusta*, and rarely on *Pinus pinea* L. (Pinaceae). Spontaneous populations have been recorded within the spray zone of irrigation facilities, but it also grows high on trunks and into the crowns of palm trees beyond the influence of irrigation waters. Its epiphytic habitat on *P. canariensis* and *B. capitata* and distinctive morphological features are depicted in Figure 7, Photographs A–E.

***Psilotum nudum* (Psilotaceae):** *Psilotum nudum* is a terrestrial or epiphytic perennial with short-creeping, rhizoid-bearing rhizomes with aerial stems to 50 cm tall (Mickel & Smith 2004; Diggs & Lipscomb 2014). It is widespread in warm-temperate, subtropical, and tropical regions of the Americas, Asia, Africa, Australia, New Zealand, and the Pacific Islands (Thieret 1993; Mickel & Smith 2004). *Psilotum nudum* grows in low mesic woods, rain forests, wet montane forests, rocky slopes, thickets, swamps, hammocks, old logs, or epiphytic at the bases of trees, tree forks, and in leaf detritus in urban gardens, 0-1,500 m elevation (Thieret 1993; Mickel & Smith 2004; Diggs & Lipscomb 2014). It is also a minor greenhouse weed (Thieret 1993).

In the United States and its territories, *P. nudum* occurs in North Carolina south to Florida and west to Arkansas, Louisiana, Texas, and Arizona, and in Puerto Rico, the Virgin Islands, and Hawaiʻi (Thieret 1993; Imada 2012; Diggs & Lipscomb 2014; Weakley 2015; USDA, NRCS 2018). In California, *P. nudum* apparently was introduced, likely on root masses as subterranean gametophytes (Farrar 2018). It is cultivated occasionally in greenhouses (CCH 2018). Although *P. nudum* has been reported previously by several authors (Roberts et al. 2004; Clarke et al. 2007; Dean et al. 2008; Roberts 2008), it has been omitted for California by the USDA, NRCS (2018).

Representative voucher specimens (duplicates to be distributed; data for many additional collections available at Consortium of California Herbaria)—**U.S.A.: CALIFORNIA: Los Angeles Co.:** City of Los Angeles, San Pedro, West 37th St. at South Dolphin St., 33.71484N -118.30736W, elev. ca. 44 m, locally abundant, epiphytic on the lower trunk of *Phoenix canariensis,* growing with *Nephrolepis cordifolia*, urban landscape, irrigation spray present, 26 Mar 2016, *Riefner 16-79* (UC). **Orange Co.:** City of Costa Mesa, E side of Orange County Fairground, Lot #A-5, W of Newport Blvd. and N of Fair Dr.,

33.66540N -117.89807W, elev. ca. 32 m, epiphytic on lower trunks of *Syagrus romanzoffiana* in irrigated planters, 22 Nov 2015, *Riefner 15-453* (UC). **San Diego Co.:** City of La Jolla, Village of La Jolla, Kline St. at Herschel Ave., 32.84387N -117.27260W, elev. ca. 38 m, epiphytic on lower trunk of *Syagrus romanzoffiana* in irrigated urban landscape, 14 Jan 2017, *Riefner 17-26* (UC).

The known epiphytic occurrences documented for *P. nudum* outside of cultivation in southern California are depicted in Figure 6. All documented populations are associated with irrigation facilities, where it grows at the base of *Phoenix canariensis* or *Syagrus romanzoffiana.*

Cyrtomium falcatum **(Dryopteridaceae): First Report for Epiphytic Behavior:** *Cyrtomium falcatum* is terrestrial or epilithic in its native and introduced range, but it also grows epiphytically in southern California's urban forests. We regard it as an accidental epiphyte, following the classification scheme of Zotz (2016).

Cyrtomiums are terrestrial, evergreen, perennial ferns native to tropical and subtropical regions distributed primarily in East Asia, but centered in southwest China (Zhang & Barrington 2013). The genus *Cyrtomium* is taxonomically difficult and not easily separated from *Polystichum* (Mickel & Smith 2004; Diggs & Lipscomb 2014). In the wild, species of *Cyrtomium* grow in soil or on rocks, not as epiphytes; a few facultative epiphytic species are known for *Polystichum* (Hoshizaki & Moran 2001; Zotz 2016). *Cyrtomium falcatum* is native to the coastal lowland forests of Indochina, Japan, Korea, and Polynesia (Zhang & Barrington 2013).

Widely escaped from cultivation, *C. falcatum* has naturalized in Europe, the Azores and Macaronesia, North America, Hawai'i, Madagascar, Reunion, South Africa, Australia, New Zealand, and elsewhere (Roux 2011; Imada 2012; Zhang & Barrington 2013; Randall 2017; ABRS 2018; Euro+Med PlantBase 2018; NZPCN 2018; USDA, NRCS 2018). In the continental United States, *C. falcatum* has naturalized primarily in the southeast, but occurrences have been reported for the Pacific Northwest, Mid-Atlantic, and Northeastern States (Yatskievych 1993; Weakley 2015; USDA, NRCS 2018). It grows on urban masonry, mesic cliffs, ravines, and rock outcrops, coastal bluffs, riparian habitats, and on streambanks (Smith 2012a; Diggs & Lipscomb 2014; Weakley 2015). For California, DiTomaso and Healy (2007) described it as occasionally naturalized without significant ecological or economic damage, possibly under-collected or overlooked. *Cyrtomium falcatum* now has been documented from diverse habitats over a broad geographical range for California (Smith 2012a; CCH 2018).

Cyrtomium falcatum is apomictic and can produce genetically variable progeny (Lollyd & Davis 1994; Smith 2012a). Juvenile sporophytes of apogamous species mature quickly, which can be advantageous in dry regions with a short growing season (Robinson et al. 2010). Thus, colonization of new sites, including water-stressed habitats, is facilitated by apogamous reproduction (Grusz 2016). During this study, small and somewhat fleshy fertile plants (<15 cm fronds) were collected on palm trunks and coastal bluffs (*Riefner 18-37*, *18-106*, *18-109*; UC). BGCI (2018) listed 'Maritimum' and other miniature *C. falcatum* cultivars that might be referable to these populations. However, we cannot, with certainty, assign a name to these plants; 'dwarfness' may be dictated by extreme growing conditions in a Mediterranean climate—a niche facilitated by the apogamous breeding system of the species.

Representative voucher specimens (duplicates to be distributed; data for many additional collections available at Consortium of California Herbaria)—**U.S.A.: CALIFORNIA: Los Angeles Co.:** City of Malibu, Eastern Malibu, N side Pacific Coast Highway at Carbon Mesa Rd., W of Carbon Canyon Rd., 34.03905N -118.6539W, elev. ca. 31 m, rare epiphyte on *Phoenix canariensis,* growing with *Nephrolepis cordifolia*, urban landscape, irrigation spray present, 13 Dec 2015, *Riefner 15-471* (UC). **Orange Co.:** City of Newport Beach, Corona del Mar, along Marguerite Ave. near Seaview Ave., 33.59523N -117.87203W, elev. ca. 30 m, epiphytic on upper *Phoenix canariensis* tree trunk, uncommon,

growing with *Ficus rubiginosa*, beyond the influence of irrigation waters, 27 Aug 2015, *Riefner 15-365* (photographic documentation); City of Laguna Beach, Aliso Beach Park, W of Coast Highway, 33.50984N -117.75135W, elev. ca. 4 m, dwarf plants uncommon, fleshy, epiphytic on trunks of *Phoenix canariensis* cultivated along parking lot, 24 Mar 2018, *Riefner 18-37* (UC). **San Diego Co.:** City of Del Mar, Coast Blvd. at 21st St., 32.96690N -117.267585W, elev. ca. 4 m, epiphytic on upper *Phoenix canariensis* tree trunk, uncommon, growing with *Solanum* sp., beyond influence of irrigation waters, 1 Feb 2017, *Riefner 17-50* (photographic documentation); City of Carlsbad, N side of Agua Hedionda Lagoon, along Adams St., E of Highland Dr., 33.14519N -117.32581W, elev. 15 m, uncommon, epiphytic on the trunks of *Phoenix canariensis*, cultivated street trees, 30 Jun 2017, *Riefner 17-324* (UC).

The epiphytic occurrences documented for *C. falcatum* in southern California are depicted in Figure 6. Populations have been recorded mostly within the spray zone of irrigation facilities, but plants also grow on upper palm tree trunks beyond the influence of irrigation waters. Its epiphytic habitat on *Phoenix canariensis* and distinctive morphologies are depicted in Figure 8, Photographs A–D.

DISCUSSION

Until recently, urban ecosystems have received little attention from scientists (Daily & Ehrlich 1999). Urban forests are one of the most complex and dynamic components of urban ecosystems (Chen & Jim 2008), yet their biodiversity remains poorly studied (Alvey 2006; Nagendra & Gopal 2011; Threlfall et al. 2016). Thus, urban ecology is a rapidly emerging discipline and urban ecosystems have become legitimate topics for study (Pickett et al. 2001).

For southern California, we document eight epiphytic fern species reproducing spontaneously in urban settings. Our findings suggest that urban forest ecosystems are promoting non-native epiphytes by facilitating interactions between a preferred cultivated phorophyte, *Phoenix canariensis*, and several epiphytic ferns escaping cultivation. Urban irrigation waters, supplemented by coastal fog drip, provide moisture during the dry summers, which in part, enable the subtropical-tropical ferns escaping cultivation to establish and disperse spontaneously in arid southern California. With recurrent severe drought, escalating costs, and inherent cut-backs for irrigation waters, the epiphytic ferns documented here are primarily species adapted to drought-prone environments.

***Phoenix canariensis*: The Preferred Urban Forest Phorophyte**

The abundance and species richness of vascular epiphytes is often higher on palms than on non-palm tree hosts (Akinsoji 1990; Aguirre et al. 2010). *Phoenix canariensis* and other species of palms with marcescent leaf bases (withering but not falling off the trunk) promote the accumulation of detritus (i.e., arboreal humus) in axils of old leaf bases that favor epiphyte colonization (Putz & Holbrook 1989; Kramer 2011; Bhatt et al. 2015). See Wagner et al. (2105) for a review.

In Mediterranean climates, which are characterized by low irregular annual rainfall and high temperatures (mild wet winters and hot dry summers), vascular epiphytes can be scarce (Torrecillas et al. 2013). However, in semiarid climates some species of palms are known to support epiphytes, such as *Phoenix canariensis* or *P. dactylifera* (Torrecillas et al. 2013). In its native habitat, the Canary Islands, *P. canariensis* is a well-known host that supports an astonishing diversity of epiphytes (Morici 1998). Interestingly, Morici (1998) also noted that *P. canariensis* trees cultivated along the historic Palm Avenue in urban Camino Largo, La Laguna, Tenerife, are also colonized by epiphytes. In addition, Brandes (2007) observed that *P. canariensis* is an important phorophyte for epiphyte colonization along the Mediterranean Sea: i.e., Gozo, Malta; Dalmatia, Croatia; Italy; and Sousse, Tunisia.

Phoenix canariensis tolerates a wide range of conditions, and worldwide is one of the most widely cultivated of all palms (Morici 1998; Zona 2008). It is also popular in southern California (Hatch 2007; Ritter 2011). Based on previous studies (Riefner & Smith 2015; Riefner 2016), reports from other Mediterranean regions, host tree architecture that promotes the accumulation of detritus and thus microhabitat resource availability, the frequency of cultivation, and the data complied during this study point to *P. canariensis* as the preferred urban forest phorophyte in coastal southern California.

Spontaneous Epiphytic Ferns: Casual Urban-Dwellers or Potential Invasive Species

Worldwide, one of the most important pathways for the introduction and dispersal of invasive plants is escape from horticulture (Reichard & White 2001; Dehnen-Schmutz et al. 2007; Faulkner et al. 2016). Urban gardens contain numerous non-native plants that are offered for sale from the ornamental horticulture industry (Raloff 2003; Pergl et al. 2016). Although most ornamentals can survive only where intentionally cultivated, some escape without human assistance (Mayer et al. 2017). Thus, cities are often hotspots of introduction and invasion where non-native ornamental plants escape gardens and disperse to surrounding environments (Gaertner et al. 2017; Mayer et al. 2017). Pteridophytes are no exception.

The eight non-native epiphytic ferns documented in this study are cultivated (Hoshizaki & Moran 2001). Four of them, *Cyrtomium falcatum*, *Nephrolepis cordifolia*, *Rumohra adiantiformis*, and *Sphaeropteris cooperi* are popular cultivated ferns (Brenzel 2012; TFF 2018). Ornamental ferns escaping cultivation disperse spontaneously by natural means, primarily by wind-blown spores originating from mature sporulating plants. Thereby, ferns introduced by horticulture and escaping gardens may represent an important colonization pathway for the epiphytic invasion of southern California's urban forests.

Although some non-native species thrive in urban habitats, they may not spread beyond city environments and/or impact biodiversity and ecosystem functions of native ecosystems (Cadotte et al. 2017). *Nephrolepis cordifolia* (Riefner & Smith 2015) and *Sphaeropteris cooperi* (Clark & Summers 2013), for example, were first reported growing outside of cultivation from California's native plant communities. However, both species volunteer spontaneously and are more widely distributed in urban environments than native habitats. Conversely, *Cyrtomium falcatum* is well-documented from native habitats (Smith 2102a), but rarely reported from urban environments (CCH 2018).

At numerous urban forest sites in southern California, aerosol sprays from irrigation facilities benefit ferns growing on the lower trunks of palm trees. However, *Cyrtomium falcatum*, *Nephrolepis cordifolia*, and *Sphaeropteris cooperi* flourish on upper palm tree trunks well beyond the influence of irrigation waters. For the summer-dry Mediterranean California climate, this epiphytic treetop habitat seemingly is unsuitable for ferns native to mesic or wet climates. Clark and Summers (2013) suggested a persistent marine layer and fog drip may have facilitated the unlikely naturalization of *Sphaeropteris cooperi* on Catalina Island cliffs in an area of relatively high exposure with little apparent moisture. Much like the cliff habitat, the epiphytic ferns documented on upper trunks of palm trees grow in a microclimate characterized by little apparent moisture and the desiccating effects of summer heat.

Characteristic fogs and overcast along the California coast, colloquially known as 'June gloom' (Wikipedia 2018b), are consistently associated with coastal Mediterranean regions where the fog season coincides with the dry season (Schemenauer & Cereceda 1991; Burgess & Dawson 2004; Fischer et al. 2009). Persistent summer fog can increase available moisture and low stratus clouds provide shade that reduces evapotranspiration and solar radiation, which alleviate plant drought stress during the rainless summers (Burgess & Dawson 2004; Williams et al. 2008; Fischer et al. 2009; Chung et al. 2017). These and other variables such as host tree architecture and bark characteristics (Wagner et al. 2015), and the nutrient status of arboreal humus (Putz & Holbrook 1989) also influence epiphyte performance.

Globally, few obligate epiphytes are invasive (Zotz 2016; Randall 2017). Invasiveness is a feature of some non-native organisms having life history traits and modes of reproduction that enable them to overcome normal barriers to invasion (Richardson et al. 2011). In Table 2, we compare our preliminary epiphytic classifications with drought-tolerance traits, native habitat occurrences, and the Global Risk Score for each species listed by Randall's (2017) *Global Compendium of Weeds*. None of these epiphytic ferns has been rated for California by Cal-IPC (2018).

Table 2. Comparison of California Adventive Ferns for Observed Epiphytic Class, Drought Tolerance, Native Habitat Occurrences, and Invasive Risk Potential.

Species	Epiphytic Class	Drought Tolerance	Documented Native Habitat(s)	Global Risk Score (GRS)
Davallia solida	Obligate	Sheds fronds or parts of blades during dry seasons	Wildland-urban interface epiphyte	—
Phlebodium aureum	Obligate	Sheds fronds seasonally during drought	—	GRS: 2.88 Rating: Low
Phlebodium pseudoaureum	Obligate	Sheds fronds seasonally, desiccation-tolerant gametophytes	—	—
Rumohra adiantiformis	Obligate	Coriaceous fronds resist desiccation	—	—
Sphaeropteris cooperi	Facultative	Unknown	Cliff and rock outcrops, wildland-urban interface epiphyte	GRS: 4.32 Rating: Low
Nephrolepis cordifolia	Facultative	Water stored in tubers, vegetative reproduction by wiry stolons	Cliff and rock outcrops, terrestrial in riparian habitats, wildland-urban interface epiphyte	GRS: 9.60 Rating: Medium
Psilotum nudum	Facultative	—	—	—
Cyrtomium falcatum	Accidental	Apomictic reproduction, precocious maturation of dwarf plants in harsh habitats	Cliff and rock outcrops, terrestrial in riparian habitats, littoral zones	GRS: 5.76 Rating: Low

Jones et al. (2018) conducted a global assessment for terrestrial non-native ferns to determine species' traits that influence naturalization and invasion. They identified 11 species of concern, including four species that we document here, namely *Cyrtomium falcatum*, *Nephrolepis cordifolia*, *Phlebodium aureum*, and *Sphaeropteris cooperi*.

CONCLUSIONS

Urban ecosystems are often underexplored, but they contain some of the most unique and interesting assemblages of species observed anywhere around the world (Francis & Chadwick 2015). The study of biological invasions, however, has only recently paid attention to urban environments (Jarošík et al. 2011; van Ham et al. 2013; Gaertner et al. 2017). Accordingly, the rising interest in urban ecosystems has been accompanied by a global surge in scientific publications and a paradigm shift in attitude—from the urban as unnatural, to the urban as interesting and ecologically important (Kowarik 2011; Wu 2014; McDonnell 2015; Salomon Cavin & Kull 2017).

Invasive ornamental species, in general, have received less attention when they do not directly invade natural communities (Geerts et al. 2017). In southern California, documentation of urban plant invasions has largely been ignored in favor of wildland habitat invasive species; in either case little attention has been given to the pteridophytes. Ferns have often been portrayed as mesic-loving plants. However, emerging studies documenting desiccation-tolerance of free-living gametophytes, in combination with highly dispersible spores of the sporophytes, facilitate the adaptation and colonization by ferns to new and often drought-prone habitats, including previously unexpected urban forest environments in arid southern California.

Additionally, there is a need for greater exploration of the socio-ecological-economic values for spontaneous urban plants of southern California. The aesthetically pleasing ferns should be part of this discussion (Morajkar et al. 2015). Southern California's interesting and attractive urban-dwelling ferns represent a prime subject for further research. We encourage others to explore and investigate the epiphytic ferns in our urban environments.

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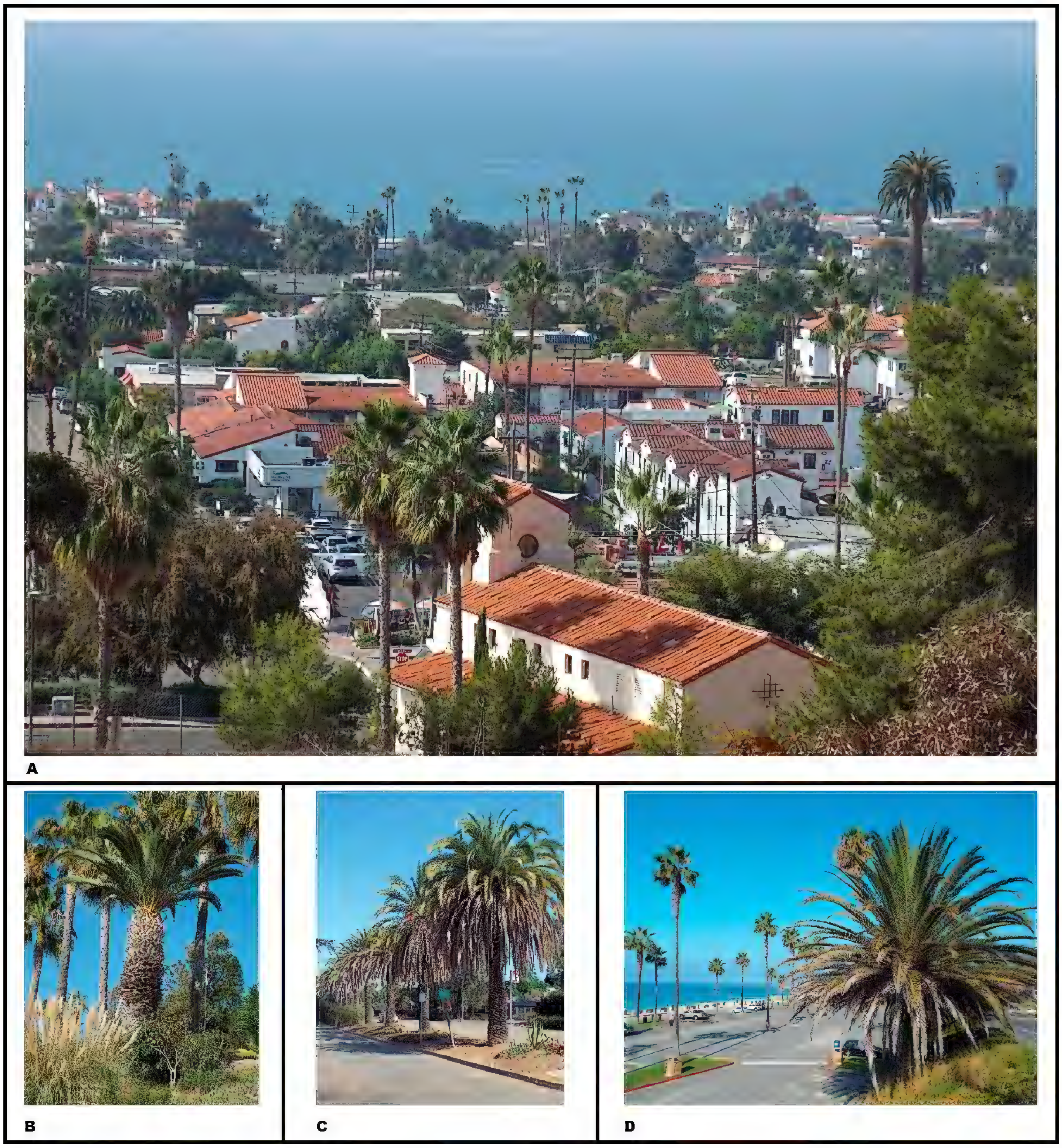

Figure 1. Urban forest, southern California: **A**–view from I-5 Freeway looking towards the Pacific Ocean showing the diversity of trees planted along city streets, business plazas, and residential housing tracts, City of San Clemente, Orange County, California; **B**–*Phoenix canariensis* and *Washingtonia robusta* cultivated in a greenbelt, City of Malibu, Los Angeles County, California; **C**–*P. canariensis* cultivated along public streets, City of San Diego, Point Loma, San Diego County, California; **D**–*P. canariensis* and *W. robusta* cultivated in coastal park, City of Laguna Beach, Orange County, California.

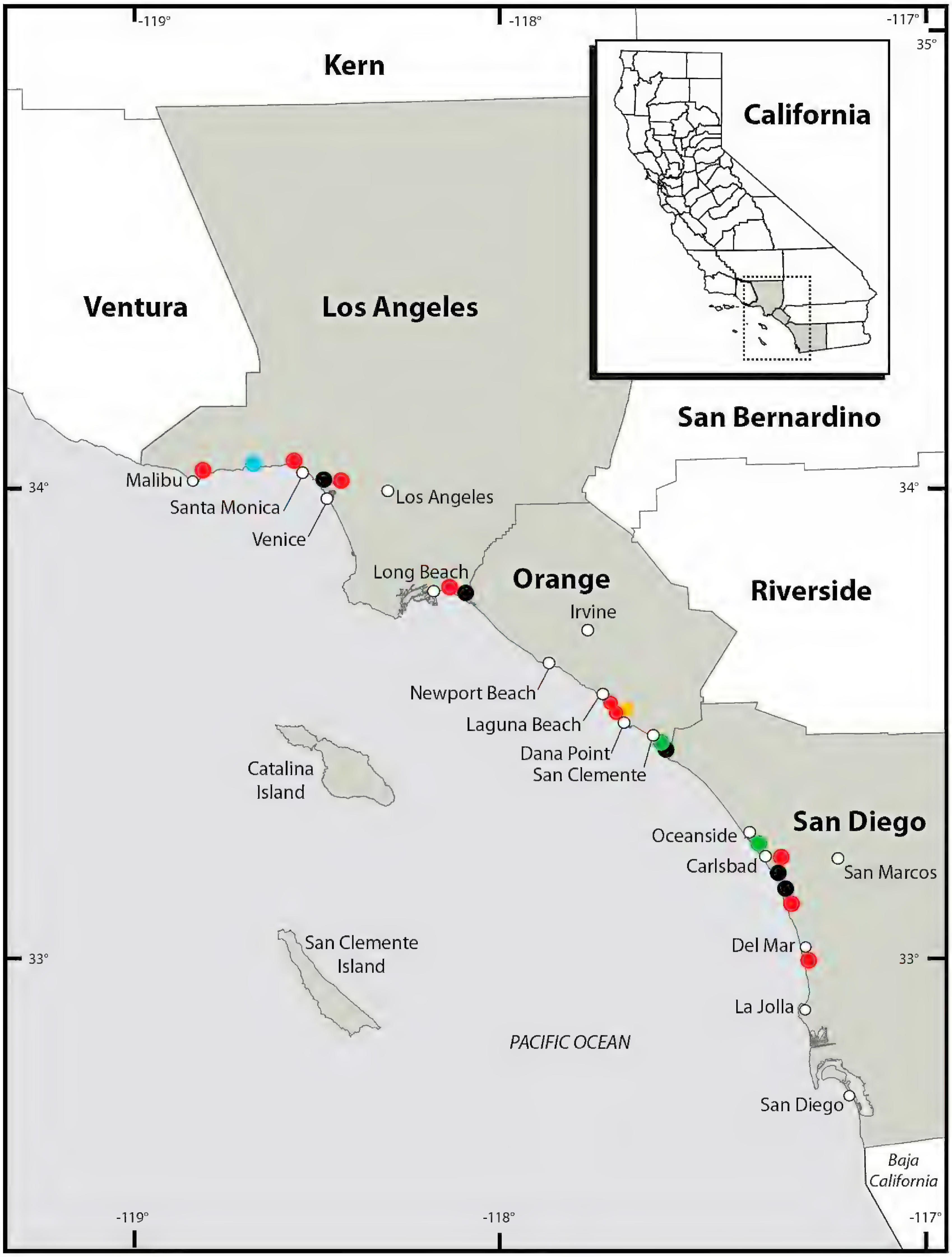

Figure 2. Known epiphytic occurrences for *Davallia solida* (●), *Phlebodium aureum* (●), *Phlebodium pseudoaureum* (●), *Rumohra adiantiformis* (●), and *Sphaeropteris cooperi* (●) documented outside of cultivation in southern California's urban forests.

Figure 3. *Davallia solida* (Oceanside, California; *RR 16-385* UC): **A**–epiphytic habitat on *Phoenix canariensis* cultivated along a public street, photographs taken during summer drought, note on far left side of trunk is drought-stressed *Nephrolepis cordifolia*; **B**–scan showing small frond size and long-creeping scaly rhizomes, scale in cm; **C**–close-up showing portions of lamina shed during periods of drought.

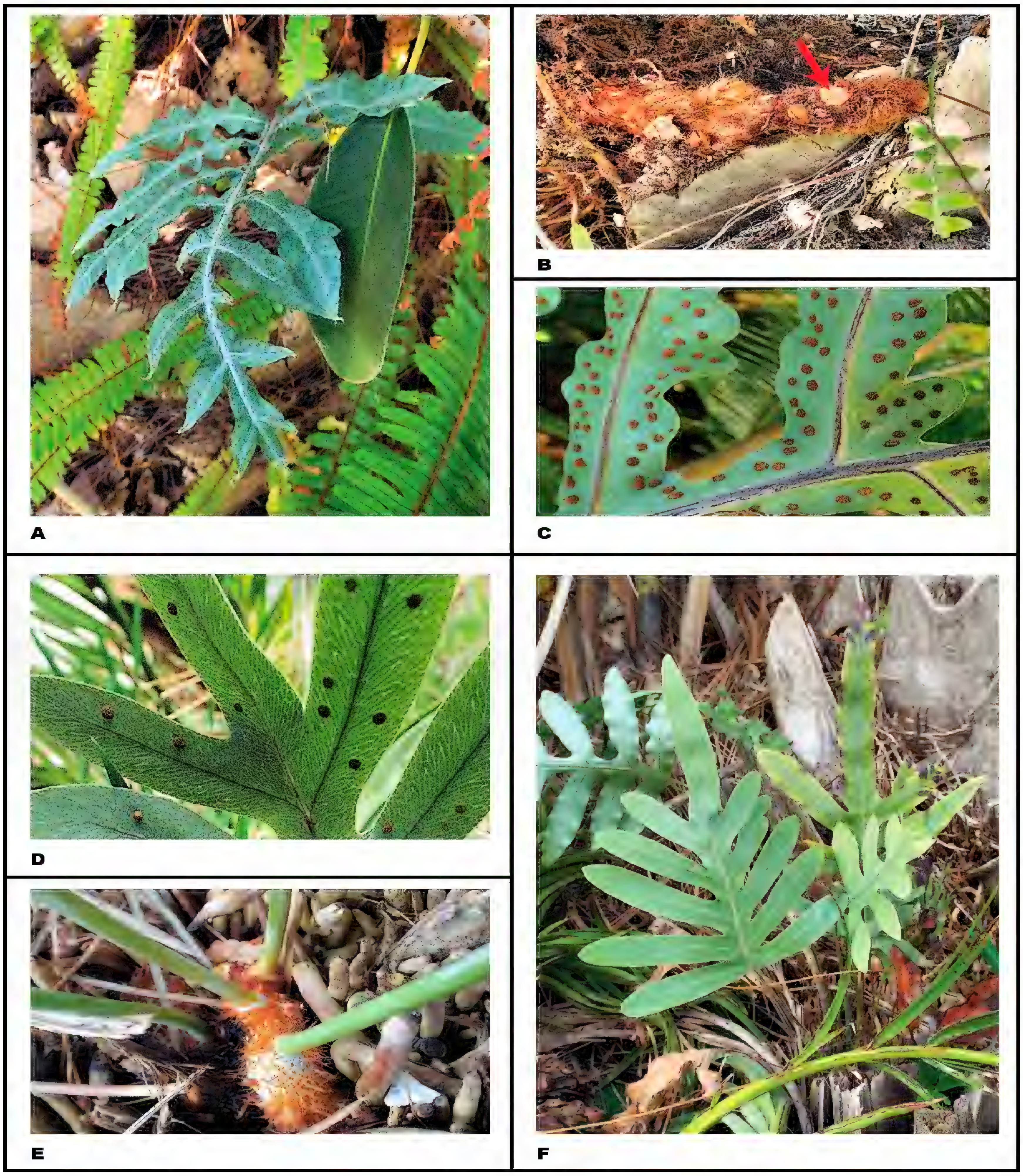

Figure 4. *Phlebodium aureum* (Malibu, California; *RR 15-469* UC): **A**–epiphytic habitat on *Phoenix canariensis* cultivated in public greenbelt, photo depicting young frond, deeply lobed with narrow segments growing with *Nephrolepis cordifolia* and *Ficus rubiginosa*; **B**–robust long-creeping rhizome with golden-brown scales, note phyllopodia (red arrow) and juvenile *N. cordifolia* frond; **C**–abaxial view of fertile frond showing sori arranged in two rows between the costa and pinna margin.

Phlebodium pseudoaureum (Laguna Beach, California; *RR 18-110* UC): **D**–abaxial view of fertile frond showing sori arranged in a single row between the costa and pinna margin and distinctive venation; **E**–creeping, pruinose rhizomes with lanceolate orange-colored scales; **F**–epiphytic habitat on *Phoenix canariensis* cultivated in park greenbelt, note deciduous fronds in lower left-hand portion of the photo.

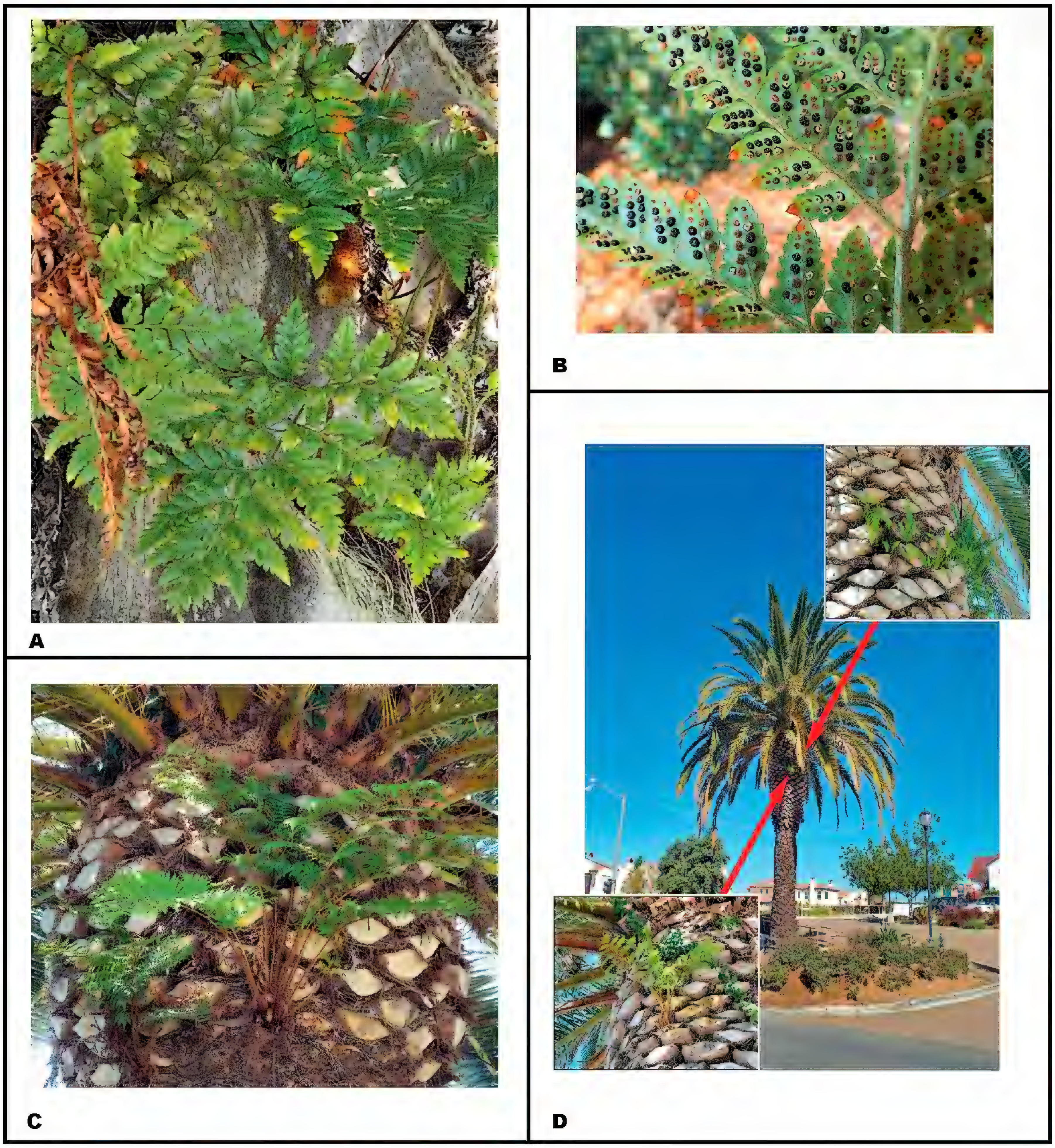

Figure 5. *Rumohra adiantiformis* (Carlsbad, California; *RR 17-52* UC): **A**–epiphytic habitat on *Phoenix canariensis* cultivated in neighborhood greenbelt; **B**–abaxial frond view showing peltate indusia that turn black and form an upside-down cup when mature.

Sphaeropteris cooperi: **C**–epiphytic habitat on upper trunk of *P. canariensis* cultivated in residential neighborhood (Laguna Beach, California; *RR 18-120* photographic voucher); **D**–epiphytic habitat on *P. canariensis* cultivated in business plaza (Carlsbad, California; *RR 15-447* photographic voucher), lower left inset close-up of upper trunk plants and upper right inset a close-up of plants growing with *Nephrolepis cordifolia* (*RR 15-448* photographic voucher).

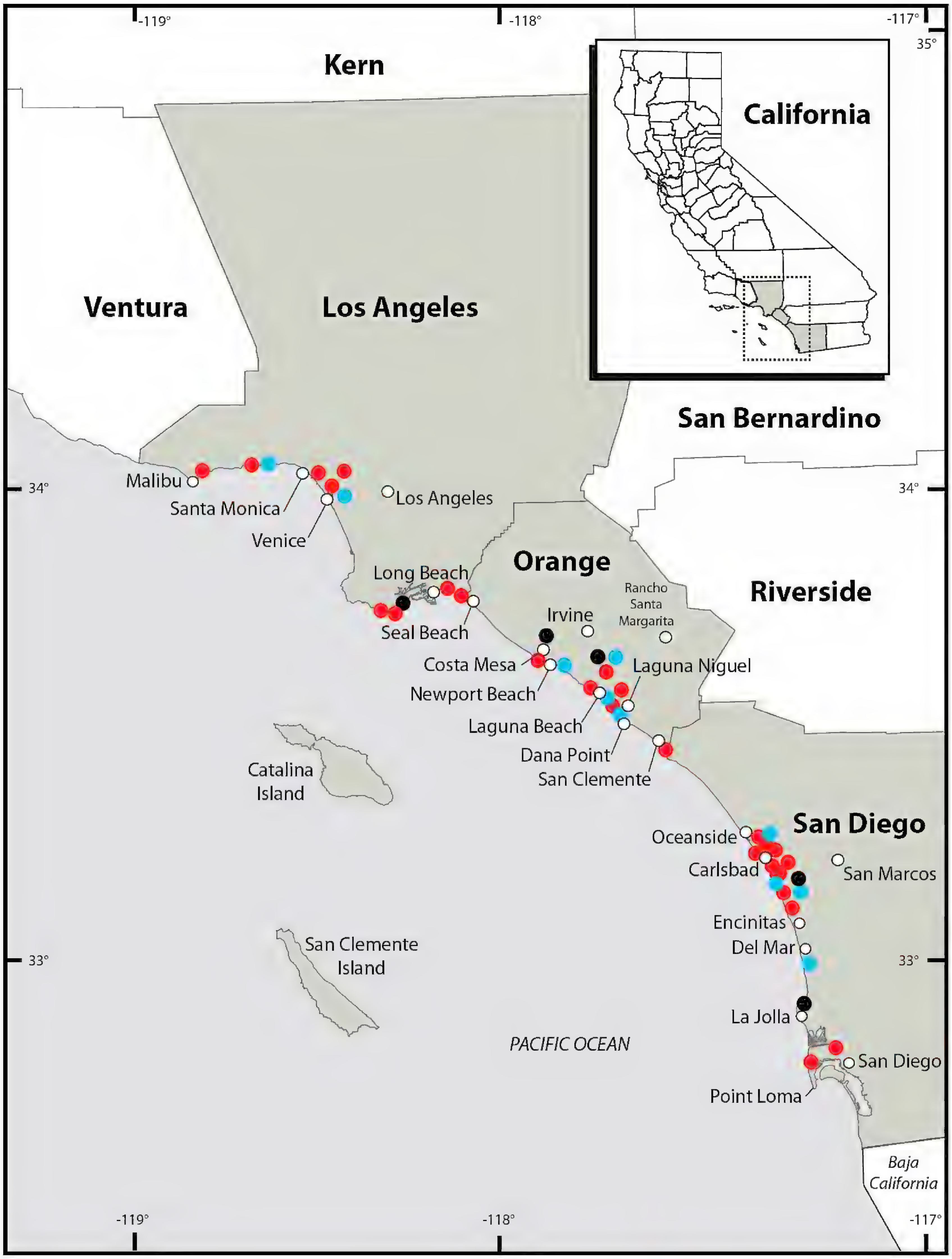

Figure 6. Known epiphytic occurrences for *Cyrtomium falcatum* (●), *Nephrolepis cordifolia* (●), and *Psilotum nudum* (●) documented outside of cultivation for southern California's urban forests.

Figure 7. *Nephrolepis cordifolia*: **A**–epiphytic habitat on upper trunk of *Phoenix canariensis* cultivated in business plaza (Mission Valley, California; *RR 17-22* photographic voucher); **B**–on *P. canariensis* cultivated in greenbelt (Santa Monica, California; *RR 17-541* UC); **C**–on *Butia capitata* cultivated along coastal promenade, red arrow points to tubers (Long Beach, California; *RR 14-258* UC); **D**–on *B. capitata* cultivated in residential neighborhood, note *Ficus microcarpa* seedling on lower right trunk (Encinitas, California; *RR 16-13* UC); **E**–on upper trunk of *P. canariensis* cultivated along public street (Point Loma, California; *RR 18-74* photographic voucher).

Figure 8. *Cyrtomium falcatum*: **A**–*Phoenix canariensis* epiphytic habitat, tree cultivated in county park greenbelt (Laguna Beach, California; *RR 18-39* UC); **B**–small size and precocious maturation may facilitate survival on palms in a Mediterranean climate (Laguna Beach, California; *RR 18-38* UC); **C**–*P. canariensis* epiphyte assemblage with *Ficus rubiginosa* on cultivated street tree, inset photo showing *C. falcatum* growing on upper palm trunk (Newport Beach, California; *RR 15-365* photographic voucher); **D**–epiphytic on upper trunk of *P. canariensis* cultivated along public street (Del Mar, California; *RR 17-50* photographic voucher).

Shortia brevistyla comb. et stat. nov., (Diapensiaceae), A narrow endemic from the headwaters of the Catawba River in North Carolina, U. S. A.

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ABSTRACT

Morphological, geographic, and molecular data justify recognition of *Shortia brevistyla* (*Shortia galacifolia* Torr. & A. Gray var. *brevistyla* P.A. Davies) as a distinct species rather than a variety of *Shortia galacifolia.* All known populations of the new *Shortia brevistyla* are found within a 10 km radius on the headwaters of the Catawba River in McDowell County, North Carolina, approximately 100 km northeast of the range of *S. galacifolia.* *Shortia brevistyla* has significantly smaller flowers, shorter styles, shorter petals, and smaller leaves than *S. galacifolia.* Genetic data indicate that the two species differ at five of 210 nucleotide positions in the ITS1 ribosomal region. Genetic divergence models indicate that the two species diverged approximately 20,000 years ago during the glacial maximum of the Pleistocene. Published on-line www.phytologia.org *Phytologia 101(2): 113-119 (June 21, 2019).* ISSN 030319430.

KEY WORDS: *Shortia brevistyla* (P. A. Davis) Gaddy, *comb. et stat. nov*., Diapensiaceae, narrow endemic, Catawba River, North Carolina.

Shortia galacifolia Torr. & A. Gray, the only species of *Shortia* heretofore known from North America (Nesom, 2009), was described by Asa Gray from a specimen in Paris collected in 1839 by Andre Michaux from the "Hautes Montagnes de Carolinie" (Williams et al. 2004). Michaux had scribbled on the herbarium sheet that the plant may be a *Pyrola* or a new genus. After extensive searches for the plant in the Carolinas, it was found to occur at low elevations (ca. 300-600 meters) along the Blue Ridge front, not in "high" mountains. Two major locations of the plant have been found in the Carolinas, one in the Keowee-Toxaway drainage of the headwaters of the Savannah River in North and South Carolina, and another on the headwaters of the Catawba River in North Carolina. Interestingly, between the areas where the plant has been found, there is a gap of approximately 100 km where no *Shortia* plants have ever been seen. Davies (1952) named the Catawba drainage plants in North Carolina "var. *brevistyla*," implying that the Keowee-Toxaway plants were "var. *galacifolia*" and concluding (as later did Zahner and Jones 1983) that the type material for *Shortia galacifolia* (in Paris) was collected from plants in the Keowee -Toxaway drainage in South Carolina (Davies 1956). Morphological, geographic, and molecular data indicate that the Catawba shortia - heretofore, *Shortia galacifolia* (Torrey & Gray) var. *brevistyla* P.A. Davies - is justifiably treated at the specific rank. It is morphologically distinct and geographically separated from the Keowee-Toxaway populations of typical *S. galacifolia*, and the two taxa have diverged significantly in nucleotide (ITS1) sequences.

METHODS

Single leaves were removed from individual *Shortia* plants sampled from both Catawba River and Keowee-Toxaway populations. Individual leaves were frozen with liquid nitrogen then ground using SPEX Sample Prep 2010 Grinder (Metuchen, NJ). DNA extractions of the leaves were performed with the Promega Wizard DNA Purification Kit (Promega Madison, WI) according to the manufacturer's instructions. Primers complementary to highly conserved regions of the ribosomal ITS1 region (Cheung 2016) were ordered from Eurofins Genomics (Louisville, KY). The corresponding region of the *Shortia* genome was amplified using PCR with the following conditions after an initial denaturation step of 4 minutes: denaturation at 94°C for 30 seconds, annealing at 55°C for 40 seconds, and elongation at 72°C for 60 seconds. After a final elongation step of 10 minutes at 72°C, a sample of the resulting PCR product was subjected to agarose gel electrophoresis to verify amplification and then sent to Eurofins Genomics for Sanger sequencing. The resulting nucleotide sequences were analyzed utilizing UGENE software (Okonechnikov 2012) and NCBI Blast (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Total DNA was extracted from dried leaf samples (ca. 0.5 cm^2) using CTAB (cetyltrimethylammonium bromide) method (Murray and Thompson, 1980) in 1.2mL tubes, after washing the leaf powder for two times with 0.8 mL of a buffer, containing 10% polyethylene glycol, 0.35M solbitol, 50mM Tris-HCl, 0.1% Bovine Serum Albumin and 0.1% β-melcaptoethanol. Genetic polymorphisms were detected with MIG-seq [multiplexed ISSR (inter-simple sequence repeats) genotyping by sequencing] technique, a PCR (polymerase chain reaction)-based procedure for constructing highly reduced representation libraries, involving *de novo* SNP (single nucleotide polymorphism) discovery, and their genotyping using high-throughput sequencing (Suyama and Matsuki, 2015). Briefly, 10 ng of DNA was used to amplify anonymous genomic regions using multiplexed PCR with tailed ISSR primers as the 1st PCR.

Eight primers of the most recommended set of MIG-seq primers (set-1) (Suyama and Matsuki 2015) were employed for the 1st PCR. PCR thermal profile was the same with the original protocol, except that the annealing temperature set to 38°C for satisfactory amplifications across samples. Subsequently, the 1st PCR products were used as templates for the 2nd PCR (tailed PCR). Using common forward and indexed reverse primers, this step permits the addition of complementary sequences for the binding sites of Illumina sequencing flow cell and index for each sample to the 1st PCR products. Then, the 42 PCR products were purified, fragments in the size range of 350–800 bp were isolated, and their final concentrations were measured by quantitative PCR. Sequencing of the multiplexed library was performed with an Illumina MiSeq Sequencer, using MiSeq Reagent Kit v3 (150 cycle, Illumina).

After read trimming using Trimmomatic v. 0.32 (with the following commands LEADING:19, TRAILING:19, SLIDINGWINDOW:30:20, AVGQUAL:20 and MINLEN:51) (Bolger *et al.*, 2014), Stacks 1.08 (Catchen *et al.*, 2011) was used to process the MIG-seq reads with the following parameter settings; the minimum number of identical reads required to create a stack ($m = 3$), the nucleotide mismatches between loci within a single individual ($M = 2$), and the mismatches between loci when building the catalogue ($n = 1$). The SNP genotype for each individual was exported with a minimum read depth of 10, using the 'populations' command. The genotype data was then processed using PLINK v. 1.07 software (Purcell *et al.*, 2007), and markers with a minor allele frequency < 0.03, missing individual rate > 0.9, missing locus rate > 0.9, and significant deviation from Hardy-Weinberg equilibrium ($P < 0.01$) were filtered out.

Population genetic statistics (the number of polymorphic loci, number of alleles, observed and expected heterozygosity, and inbreeding coefficient) were summarized for each population using GenAlEx 6.5 (Peakall and Smouse, 2012). The significance of Hardy–Weinberg equilibrium (heterozygote deficiency) were examined by exact test implemented in Genepop 4.2 (Rousett, 2008). Population

differentiation was assessed by a summary statistic of F_{ST} and AMOVA (analysis of molecular variance) using GenAlEx 6.5, and visualized by PCA (principal coordinate analysis) using '*adegenet*' package (Jombart, 2008) in R 3.1.0 (R Development Core Team 2014).

Past population demography was modeled by applying a pure isolation model (Hey and Nielsen 2007) to the MIG-seq sequence data. The population model assumed an ancestral population split into two descended populations (i.e. extant var. *galacifolia* and var. *brevistyla*) at a past time (t). These populations were allowed to have different parameters of effective population size (θ_{anc}, θ_{glc}, and θ_{brv}). In Bayesian parameter estimation using IMa2 program (Hey and Nielsen, 2007), the maximum priors for each parameter was set as follow: $t = 1$, $\theta_{anc} = \theta_{glc} = \theta_{brv} = 3$, and under this prior settings, 10 Metropolis-coupled MCMCs with a heating scheme of geometric model (with parameter specifications of -ha 0.99 and -hb 0.75) were generated for 3 million steps after a burn-in period of 1 million steps. Infinite-site model was used to model the sequence evolution of MIG-seq data. To confirm the adequate chain mixing and parameter convergence, independent two runs were performed. After obtaining the posterior parameters, demographic parameters were scaled to absolute divergence time in years and effective population size, by applying the geometric mean of 7.0×10^{-9} (substitutions/site/year) estimated for *Arabidopsis* species (Ossowski et al. 2010) and a generation time of 10 years for *Shortia.*

DESCRIPTION OF SPECIES

SHORTIA BREVISTYLA (P. A. Davies) Gaddy, *comb. et stat. nov*. (Figure 1).

Shortia galacifolia var. *brevistyla* P.A. Davies, Basionym. *Rhodora* 54: 124. 1952.

TYPE: U. S. A. North Carolina, McDowell Co., John's Creek (Fish Hatchery Creek) above the Fish Hatchery, on moist creekbank under *Rhododendron maximum*, 23 March 1951, *P. A. Davies 9149* (holotype: GH!; topotype: USCH!).

Differs from *Shortia galacifolia* Torrey & Gray in that its styles are shorter, its petals are shorter and shallowly toothed, and its leaves are smaller.

Rhizomes: slender, scale-leaved, lignescent. **Stems:** erect, unbranched. **Leaves:** basal, rosulate from rhizome buds, 20-55 mm in length; petiole present; blade orbiculate to elliptic-orbiculate, ovate-oblong, or ovate, margins coarsely crenate-serrate, apex emarginate to truncate, surfaces glabrous, pinnately veined. **Scapes**: bracteate, elongating after flowering. **Inflorescences:** solitary. **Flowers:** sepals distinct; petals 12-17 mm long, corolla rotate to campanulate, lobes white to pink to faded rose-purple, margins slightly toothed; anthers 2-locular, without basal spurs, longitudinally dehiscent; filaments adnate to corolla tube; staminodes present. Styles 6-12 mm in length.

Davies (1952) used style length as the primary character in his description of *Shortia galacifolia* var. *brevistyla.* Although the styles *are* significantly shorter than those of *galacifolia*, styles in *S. brevistyla* populations are often variable in length. And although petals and leaves are significantly shorter in *S. brevistyla,* the best field character, however, is the shallow, blunt teeth on the petals of *S. brevistyla* versus the deeply laciniate teeth on the petals of *S. galacifolia* (Figure 1; Table 1).


Figure 1. Typical flowers of *brevistyla* (left) (2.5x) and *galacifolia* (2x).

Table 1. Morphology of *Shortia galacifolia and Shortia brevistyla.*[1]

SPECIES	FLOWER SHAPE	LEAF LENGTH (MEAN)	PETAL LENGTH	PETAL DISTAL MARGIN	STYLE LENGTH
galacifolia	campanulate	37-82 mm (54)	16-25 mm	deeply laciniate	12-18 mm
brevistyla	rotate to campanulate	20-55 mm (41)	12-15 mm	shallowly toothed	6-12 mm

[1]Data from Davies (1952), Hatley (1977), Nesom (2009), and L. L. Gaddy (field observations: 2018-19).

RESULTS AND DISCUSSION

Two independent analyses of the chromosomal ribosomal ITS1 region were performed using DNA isolated from *Shortia* plants sampled from two different locations within each of the two geographic regions in North Carolina and South Carolina—the Catawba shortia region and the Keowee-Toxaway region—where North American *Shortia* is found. In both sample sets, we obtained 210 base pairs of nucleotide sequence for each sample that was 100% identical for all the North Carolina specimens as well as the *Shortia* ITS1 sequence present in the GenBank database (Table 2). Similarly, the nucleotide sequences of the South Carolina samples were identical, but differed from the North Carolina samples at five nucleotide positions. Therefore, we concluded that the Catawba River drainage North Carolina *Shortia* is genetically distinct from the Keowee-Toxaway (Savannah River) drainage South Carolina *Shortia* and that they have been reproductively isolated from one another long enough for ITS1 regions to diverge at 2% of the nucleotide positions.

Data from the laboratories of Dr. Shota Sakaguchi revealed that after adapter and quality trimming, an average of 191.4k sequence reads were obtained for the individual samples. The reads were then assembled and filtered by Stacks 1.08 and PLINK v. 1.07, which resulted in a genotype matrix consisted of 97 SNP markers. The genotyping rate at these markers was high: 97.1% for *galacifolia* and 99.0% for *brevistyla.* Significantly lower genetic diversity in *brevistyla* is revealed in multiple statistics (Table 2). Among the 92 markers, only 19.6% showed allelic variation in *brevistyla,* while 93.4% were polymorphic in *galacifolia.* The number of alleles (Na = 1.92 in *galacifolia* vs. 1.20 in *brevistyla*) and the heterozygosity index (e.g., H_E = 0.266 in *galacifolia* vs 0.072 in *brevistyla*) were consistently low in *brevistyla.* The inbreeding coefficient values were slightly negative in both species (they are both narrow endemics) but were not significantly deviated from Hardy-Weinberg equilibrium ($P > 0.05$ for both populations) (Figure 2; Table 3).

Table 2. Divergent nucleotide positions in comparison of ITS1 regions in Keowee-Toxaway drainage (SC) and Catawba River drainage (NC) *Shortia* populations.

SC	NC	Nucleotide Number[1]
A	C	73
T	C	84
T	C	100
G	A	158
C	A	159

[1]Sequence number from AY049800, labeled "*S. galacifolia*" ITS1 sequence in Genbank (as it turns out, the Genbank sequence is from *S. brevistyla*).

Table 3. Summary genetic diversity statistics for *galacifolia* and *brevistyla.*[1]

Species		N	P	N_a	H_O	H_E	F_{IS}
galacifolia	average	21.36	0.934	1.92	0.270	0.266	-0.005
(n=22)	SE	0.09	--	0.03	0.018	0.015	0.026
brevistyla	average	19.78	0.196	1.20	0.095	0.072	-0.256
(n=20)	SE	0.04	--	0.04	0.024	0.017	0.034

[1]N: number of genotyped individuals, P: proportion of polymorphic loci, N_a: number of alleles, H_O: observed heterozygosity, H_E: expected heterozygosity, F_{IS}: inbreeding coefficient.

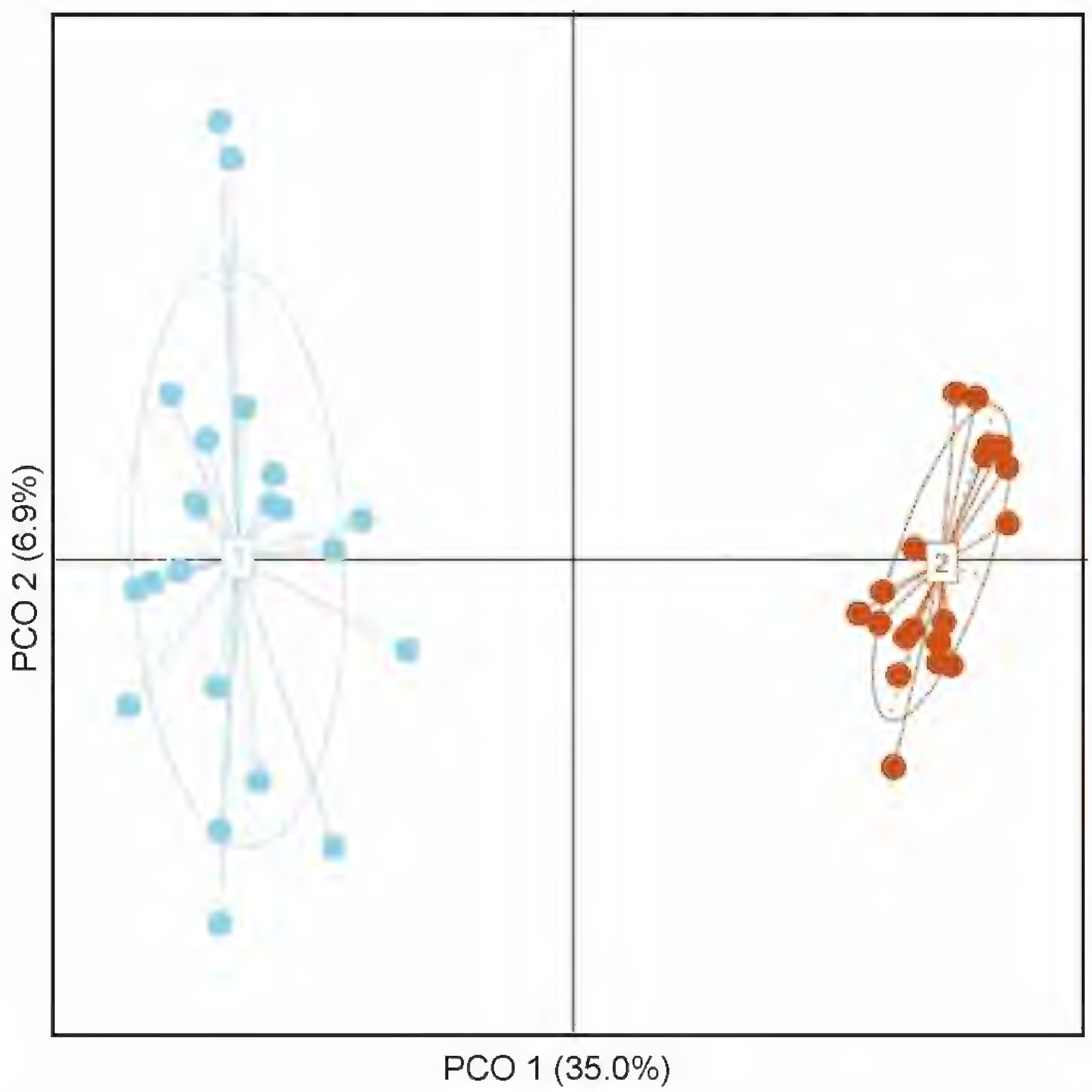

Figure 2. Plot of principal coordinate analysis scores of individual samples: 1-*galacifolia*; 2-*brevistyla.* Numbers on the coordinate axes are the percent variance accounted for by that axis.

The independent two runs using IMa2 program gave highly consistent posterior distributions of demographic parameters, indicating the robustness of our Bayesian analyses. The estimated effective population size of was 5396 (2211, 9641) for *galacifolia* and 619 (265, 2388) for *brevistyla*, respectively [HiPt (95% low, high) values are shown as posterior distribution summary]. The larger population size estimated for the common ancestral population [20609 (15302, 28216)] showed the extant two "species" experienced demographic decline after lineage divergence. The divergence event was estimated to have occurred 20049 (8255, 53070) years before present, a timing roughly corresponding to the last glacial maximum of the Pleistocene.

Like other species of *Shortia* (Gaddy and Nuraliev 2017), both North American species of the genus are narrow endemics with very small ranges and exacting habitats. All the known populations of *S. brevistyla* are all found within 10 km radius in the upper Catawba River drainage just north and northeast of Marion, North Carolina in McDowell County. The more numerous populations of *Shortia galacifolia* all fit into an area within a 15 km radius around its type locality in the Keowee-Toxaway drainage just north of the Lake Jocassee dam (in Oconee County, South Carolina). Finally, the southwestern most *S. brevistyla* population known is about 100 km northeast of the northeastern most *S. galacifolia* population.

Shortia brevistyla appears to have speciated due to genetic drift in isolated populations of *Shortia galacifolia* with low heterozygosity and self-pollinating plants. Estimates, using Bayesian estimation in the IMa2 program, indicate that these two species diverged approximately 20,000 years ago during the time of the glacial maximum (Osssowski et al. 2010).

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Mass Spectral identification and quantitation of Single Ion Chromatography of unresolved limonene, sylvestrene and β-phellandrene in the leaf volatile terpenoids of *Pinus peuce* (Bulgaria) and comparison with oils from other regions

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ABSTRACT

Analyses of the leaf volatile oils of *Pinus peuce* from Bulgaria revealed the oil is dominated by α-pinene (27.1-49.2%), camphene (2.8-8.6%), β-pinene (9.7-20.4%), and germacrene D (8.7-10.6%). Sylvestrene varied considerable from 1.5 to 34.9%. Two plants were very high (12.5, 34.9%) and three plants were low in sylvestrene (1.5, 1.9, 2.0%), suggesting chemotypes are present. Comparisons with *P. peuce* from Montenegro-Serbia, Serbia, Macedonia and Greece showed the compositional averages appeared to be fairly uniform in the Balkans, except for the literature reports of limonene, sylvestrene and β-phellandrene. Bulgaria α-pinene (38.8%) is similar to oils from other regions (Table 1), although Karapandzova et al. (2011) reported a population with only 9.2% α-pinene. Camphene (6.1%) was similar to other regions. β-pinene was variable among regions with Bulgarian oil being high (16.2%), but not as high as reported from Greece (22.0) by Koukos et al. (2000). The amounts of limonene, sylvestrene and β-phellandrene were inconsistent (Table 1) and this probably reflects both the difficulty of identification and the lumping of compounds into one value in some reports. Published on-line www.phytologia.org *Phytologia 101(2): 120-130 (June 21, 2019)*. ISSN 030319430.

KEY WORDS: *Pinus peuce,* Bulgaria, volatile leaf oil, terpenes, composition, mass spectra limonene, sylvestrene, β-phellandrene, Single Ion Chromatograms.

The advances in capillary column technology have made the analysis and identification of volatile oil components from plants relatively easy (Adams 2007). The most commonly used capillary column is bonded, 5% phenyl 95% dimethylpolysiloxane due to its excellent resistance to degradation and range of use from ambient to 325˚ C (350˚C max). When I (Adams) began to assemble a terpene library in 1976 in Dr. Ernst von Rudloff's laboratory, we used only carbowax columns (PEG, polyethylene glycol) which were short lived due to degradation by oxygen and water. The advent of bonded, 5% phenyl 95% dimethylpolysiloxane capillary columns was championed by Walter Jennings who co-founded J & W Scientific in 1974. Soon J & W released the DB-5 column (Duro Bond 5% phenyl 95% dimethylpolysiloxane). DB-5 is equivalent to: Rtx-5 (Restek), G27, G36 (USP nomenclature), HP-5, CP-Sil 8 CB (Agilent), BP-5 (SGE), ZB-5 (Phenomenex), Optima-5 (Machery-Nagel), SPB-5 (Supleco), EC-5, AT-5 (Alltech) and 007-5 (Quadrex). I (Adams) immediately seized on the opportunity and adopted DB-5 as the standard column for volatile oil analyses for use in building the Adams Terpene Library (Adams 2007, 4th ed.), in spite of the almost universal utilization of carbowax (PEG) columns in the fragrance and flavors industry at the time. No column is capable of resolving all components in all volatile oils. Systems are now available for simultaneous co-chromatography on a non-polar (ex. DB-5) and a polar (ex. PEG) column. But, a vast number of labs still use DB-5 (or equivalent) as their primary column. However, there is a very common, and thus, important duo or trio of compounds that co-elute: limonene, sylvestrene and β-phellandrene. They are difficult to identify and

quantify on DB-5. These three compounds are found in the leaf volatile oils of several *Pinus* species (Ioannou et al. 2014) and are sometimes just reported together (limonene-β-phellandrene, Karapandzova, et al. 2011).

Single Ion(s) Chromatograms (SIC) is a powerful tool available on mass spectrometers. SIC can be used to identify and quantitate co-eluting compounds such as limonene-sylvestrene-β-phellandrene; α-cedrene-β-funebrene; and cedrol-widdrol. In a study of *Pinus peuce* in Bulgaria, we found a tree that had limonene (trace), sylvestrene (12.5%) and β-phellandrene (0.7%) in its oil and presented an opportunity to carefully investigate the feasibility of utilizing SIC for identification and quantitation.

Pinus peuce Griseb. is endemic to the Balkan peninsula (Serbia, Montenegro, Albania, Macedonia and Bulgaria) Gymnosperm Database (2019). There are several reports of the leaf (needle) essential oil composition of *Pinus peuce* (Ioannou et al. 2014; Karapandzova et al. 2010, 2011; Koukos et al. 2000; Mitic et al. 2017; Nicolic et al. 2014; Petrakis et al. 2001. Mitic et al. (2017) has given an excellent, recent review *P. peuce* taxonomy, history and essential oil analyses, so the reader is referred to that paper.

The purposes of this paper are to report on an investigation of the feasibility of utilizing SIC for identification and quantitation of limonene-sylvestrene-β-phellandrene, and present a complete analysis of the volatile leaf of *Pinus peuce* from Bulgaria and compare its composition with reports from the Balkans.

MATERIALS AND METHODS

Leaf samples of *P. peuce* collected: Bulgaria, Pirin Mountain (North), National Park "Pirin". Between the huts "Banderitza" and "Vihren", in the valley of Banderishka river, growing with *Pinus heldreichii, P. mugo, Juniperus communis,* 41°45'57.7" N, 23°25'25.1" E., 1831 m, Date 18.05.2015. Coll. Alexander & Nikolay Tashev 2015 Sp. 1-5 PP1-P5, Lab Acc. *Robert P. Adams 14737-14741*. Voucher specimens are deposited at University of Forestry, Dept. of Dendrology, Sofia, Bulgaria.

Gently dried leaves (100g, 40 - 45˚C) were cut into 3 cm lengths to promote the release of terpene oils and then immediately steam distilled for 2 h using a circulatory Clevenger-type apparatus (Adams, 1991). The oil samples were concentrated (ether trap removed) with nitrogen and the samples stored at -20ºC until analyzed. The extracted leaves were oven dried (100ºC, 48 h) for determination of oil yields.

The oils were analyzed on a HP5971 MSD mass spectrometer, scan time 1/ sec., directly coupled to a HP 5890 gas chromatograph, using a J & W DB-5, 0.26 mm x 30 m, 0.25 micron coating thickness, fused silica capillary column (see Adams, 2007 for operating details). Identifications were made by library searches of the Adams volatile oil library (Adams, 2007), using the HP Chemstation library search routines, coupled with retention time data of authentic reference compounds. Limonene, sylvestrene and β-phellandrene eluted as a single peak on DB-5, but their amounts were quantitated by use of Single Ion Chromatograms using ions: 67, 68, 77, 79, and 93. Quantitation was by FID on an HP 5890 gas chromatograph using a J & W DB-5, 0.26 mm x 30 m, 0.25 micron coating thickness, fused silica capillary column using the HP Chemstation software.

RESULTS AND DISCUSSION

Mass spectra of pure limonene, sylvestrene and β-phellandrene are shown in Fig. 1(modified from Adams, 2007, p. 143). Colored ellipses indicate the critical mass pairs that resolve these three compounds. The following is a schematic for identification of limonene (LMNN), sylvestrene (SYLV) and β-phellandrene (BPHL):

A. Retention times (time, KI): LMNN (8.69, 1029, SYLV (8.80, 1027), BPHL (8,82, 1029). If only LMNN and BPHL are present, and neither are large components, they can sometimes be resolved, but if not resolved, **then use single ions as:**

B. Mass Ion 93: Note that pure LMNN (Fig. 1) has 68>93, but if LMNN is just a trace (as in *P. peuce, 14740*), even the 'purest' spectrum of LMNN may not have 68>93 (see Fig. 2a, where 68 is only ½ the size of 93, due to the presence of considerable sylvestrene).
However, 93 is very useful when there is partial peak separation between LMNN, SYLV, and/ or BPHL.

C. Next, consider Mass Ions 77, 79: If **77>79**, this implies BPHL is present (Fig. 1); So, check the mass spectrum in your reference library, it should be β-phellandrene (cf. Fig. 1).
If 79>77, this implies LMNN or SYLV is present as both have ion 79>77. (of course, there may be another unidentified compound present, never forget this.)

D. Check if ion 68>93 for LMNN (but this did not work for plant *14740*, because of the large amount of sylvestrene that contributes ion 93 (Fig. 1).

E. Then, check if 68>67 or if 67 ~ 68. Checking 67 vs. 68 in individual scans from about 8.680 mins thru 8.698 revealed that ion 67 gradually increased and the ratio of 67/68 increased such that the height of 67 was approximately equal to 68 (i.e, 67~68) at 8.698, as one would expect to find in sylvestrene (compare 67/68 in Fig. 2a and 2b).

F. In addition, one can check other trends of ratios: we found that the ratio of 68/93 declined from 8.680 mins thru 8.698 due to increases in the amount of sylvestrene (cf. compare 68/93 in Fig. 2a and 2b). Clearly trace amounts are very difficult to detect, but can be detected by very careful single ion analyses.

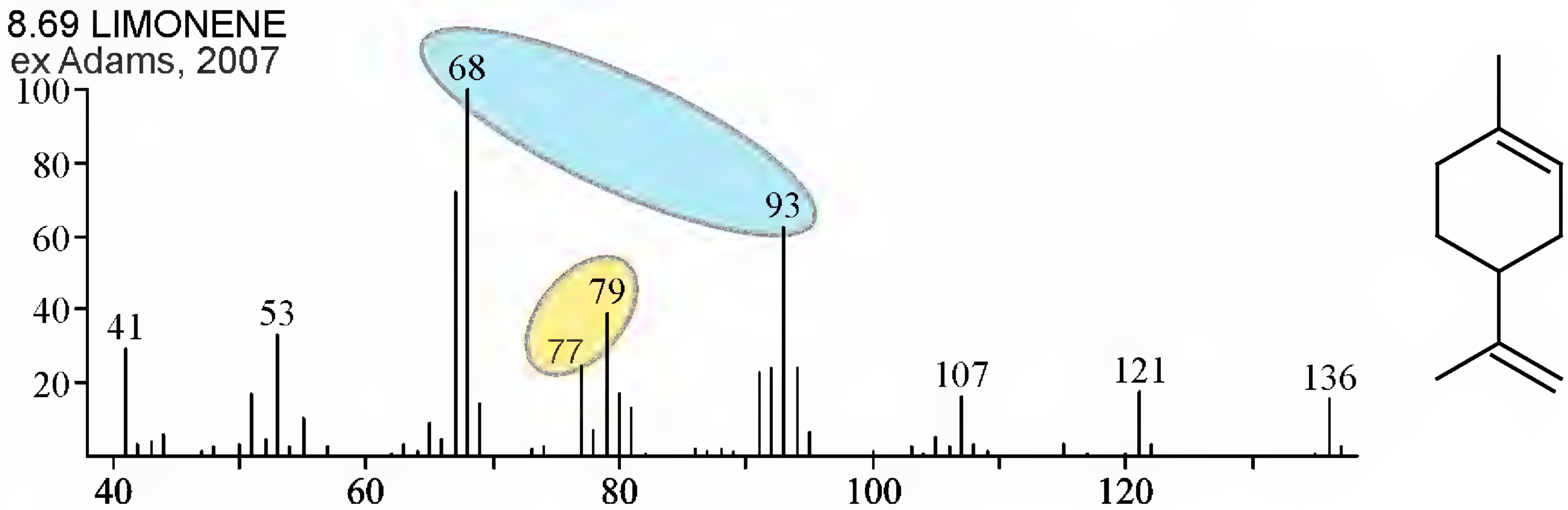
The SIC region from 8.754 to 8.809 was nearly pure sylvestrene and constitutes a large majority of the SIC peak. In fact, there is mostly sylvestrene from 8.698 thru 8.809 and it is estimated to be 95% of the peak (Fig. 2b, upper).

The amount of β-phellandrene (from 8.828 to 8.846 mins) is estimated at about 5% of the SIC peak (Fig. 2c, upper). Although these estimates of limonene, sylvestrene and β-phellandrene are just estimates, it seems prudent to utilize these mass spectral methods to report composition, rather than lump the components into one sum.

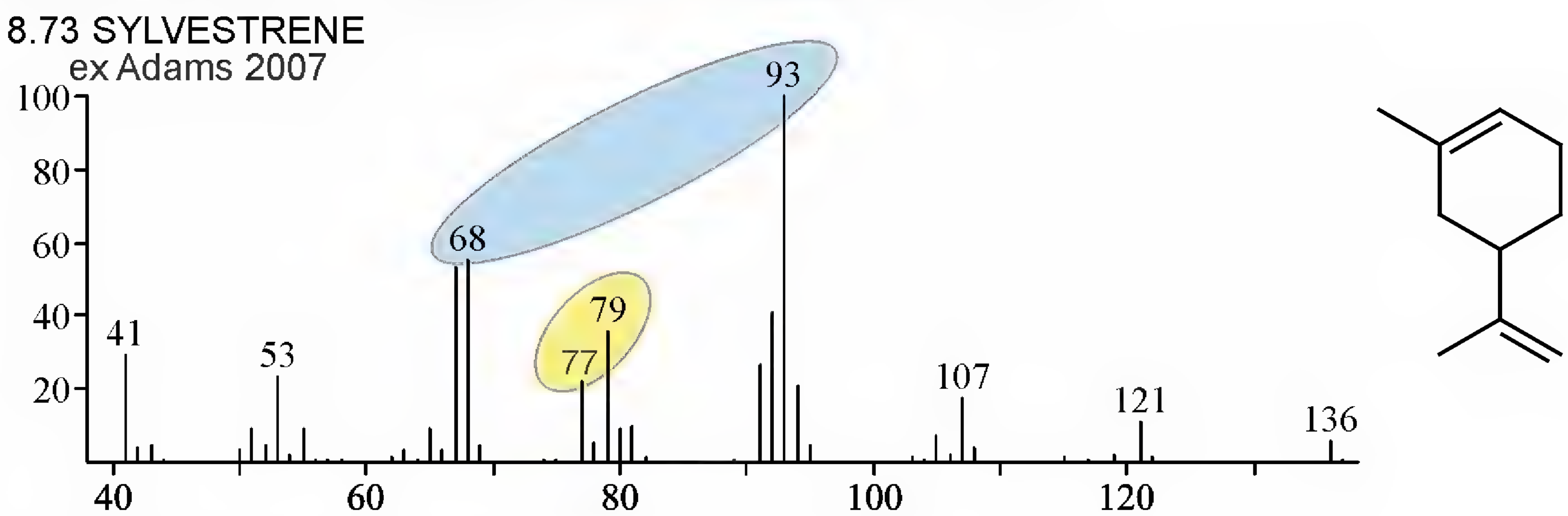
COMPARISON OF THE OIL OF *P. PEUCE* FROM BULGARIA WITH OTHER REGIONS

The composition of the volatile leaf essentials oils of *P. peuce* from Bulgaria are shown in Table 1 and compared with Montenegro-Serbia (Mitic et al. 2017), Serbia (Nikolic et al. 2014), Macedonia (Karapandzova et al. 2011) and Greece oils (Koukos et al. 2000; Petrakis et al. 2001). Bulgaria α-pinene (38.8%) is similar to oils from other regions (Table 1), although Karapandzova et al. (2011) reported a population with only 9.2% α-pinene. Camphene (6.1%) was similar to other regions. β-pinene was variable among regions with Bulgarian oil being high (16.2%), but not as high as reported from Greece (22.0%) by Koukos et al. (2000).

RT: 8.69 AI: 1024 KI: 1029 **Limonene**
CAS#: 138-86-3 MF: C10 H16 FW: 136 MSD LIB#: 164
CN: methyl-4-(1-methylethenyl)cyclohexene <1->
Synonyms: cajeputene; cinene; kautschin; nesol; p-mentha-1,8-diene; dipentene
Source: distillation of sweet anis leaves. Calif., RP Adams #5452; 96.49% *Citrus sinensis*, Zhiwu, Xuebao, 30:623(1988); 96.41% *Citrus aurantium*; 95.00% *Citrus deliciosa*



RT: 8.80 AI: 1027 KI: 1030 **Sylvestrene**
CAS#: 1461-27-4 MF: C10 H16 FW: 136 MSD LIB#: 1453
CN: cyclohexene, 1-methyl-5-(1-methylethenyl)-
Synonyms: m-mentha-1(6),8-diene; Source: ref. cpd. ex E. von Rudloff



RT: 8.82 AI: 1028 KI: 1029 **Phellandrene <β->**
CAS#: 555-10-2 MF: C10 H16 FW: 136 MSD LIB#: 161
CN: methyl-6-(1-methylethyl)cyclohexene <3->; Synonyms: p-menth-1(7),2-diene
Source: distillation of sweet anis leaves(Calif.), RP Adams #5452; 63.05% *Levisticum officinale*, p. 641, in: Flav. & Frag.: A World Persp., Lawrence, etal., Elsevier Science Publishers, Amsterdam(1988); 61.00% *Angelica archangelica*; 21.00% *Lavandula pinnata*

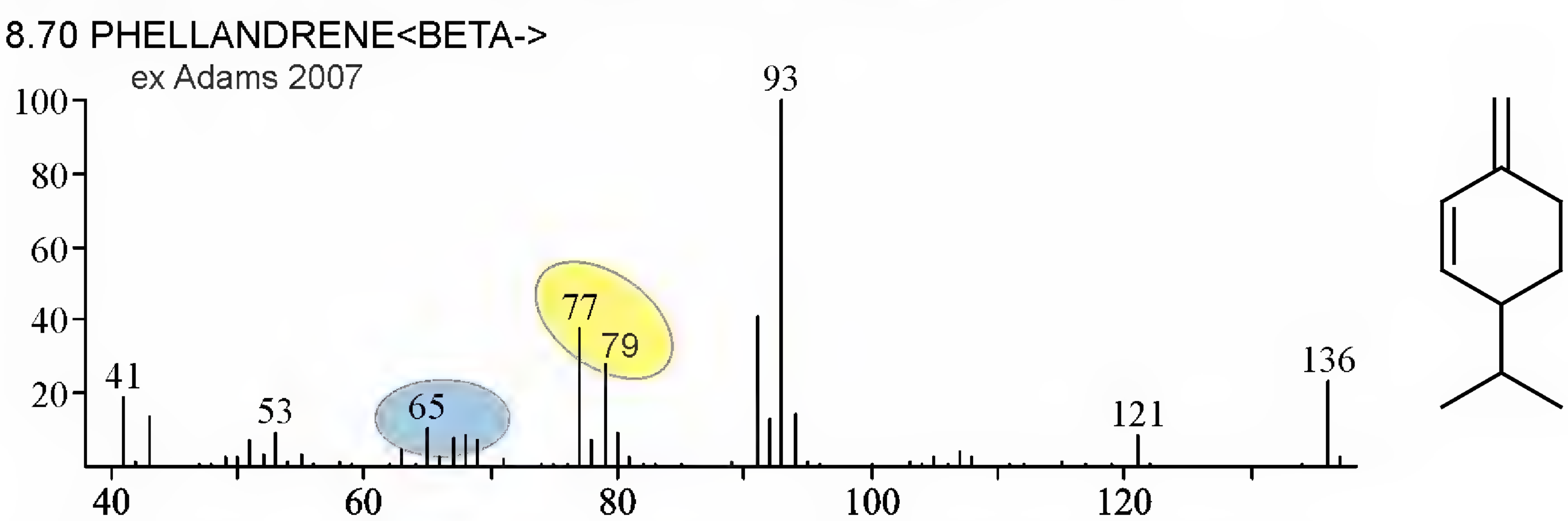


Figure 1. Mass spectra of limonene, sylvestrene and β-phellandrene (ex Adams 2006). Circles indicate diagnostic mass pairs for identification and quantification.

Fig. 2. Single Ions Chromatograms for ions 93, 79, 77, 68, and 67 (superimposed, for 8.5 - 9.0 mins.)

Fig. 2a. MS of limonene (lower section) from the average of 8.680 to 8.698 mins in SIC region as shown by the 2 arrows (upper). The MS has some sylvestrene contamination. Note the point of inflection in ion 93 at about 8.698, indicating an increase in sylvestrene.
Limonene is only a trace of the SIC peak. (cf. reference MS in Fig. 1)

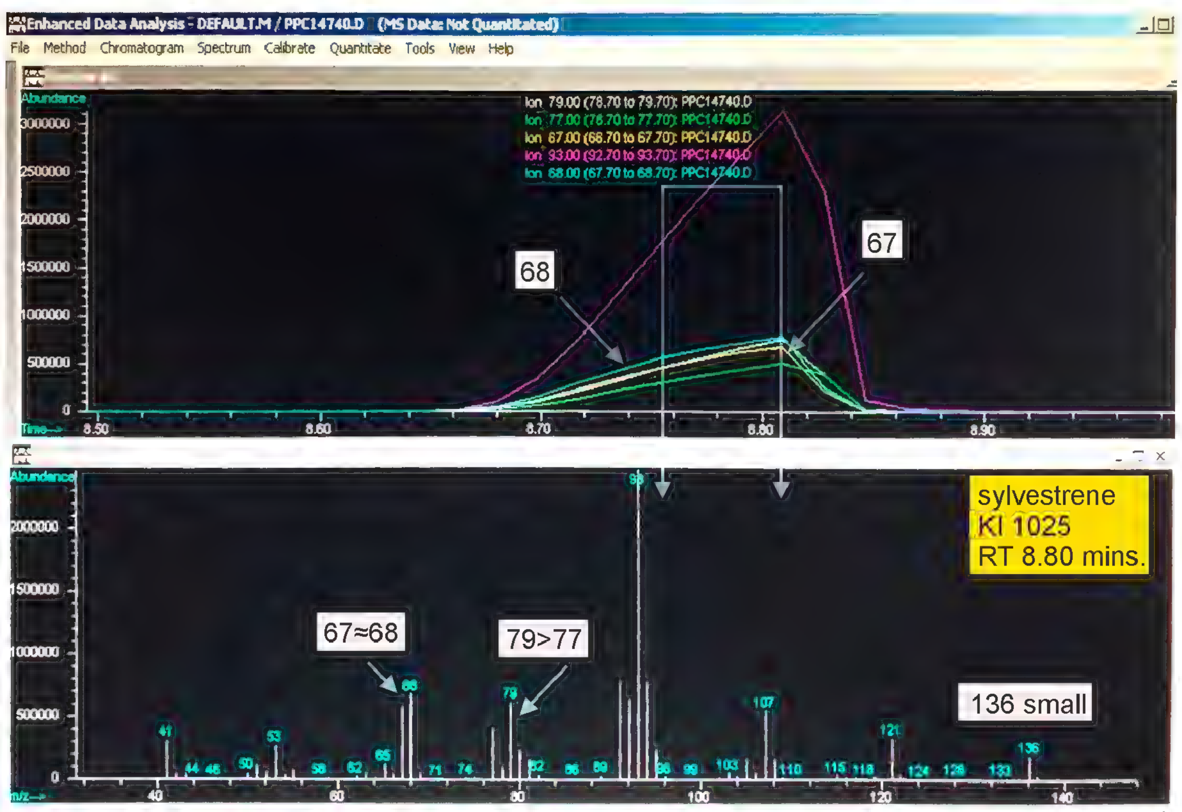


Fig. 2b. The MS of sylvestrene (lower section) from the average of 8.754 to 8.809 mins in SIC region as shown by the 2 arrows (upper). Notice (in the SIC), the maximum amounts of ions 67, 68, 79 at 8.809, revealing the peak top of sylvestrene. This is a very pure sylvestrene MS (cf. reference MS in Fig. 1.). Sylvestrene is about 95% of the SIC peak.

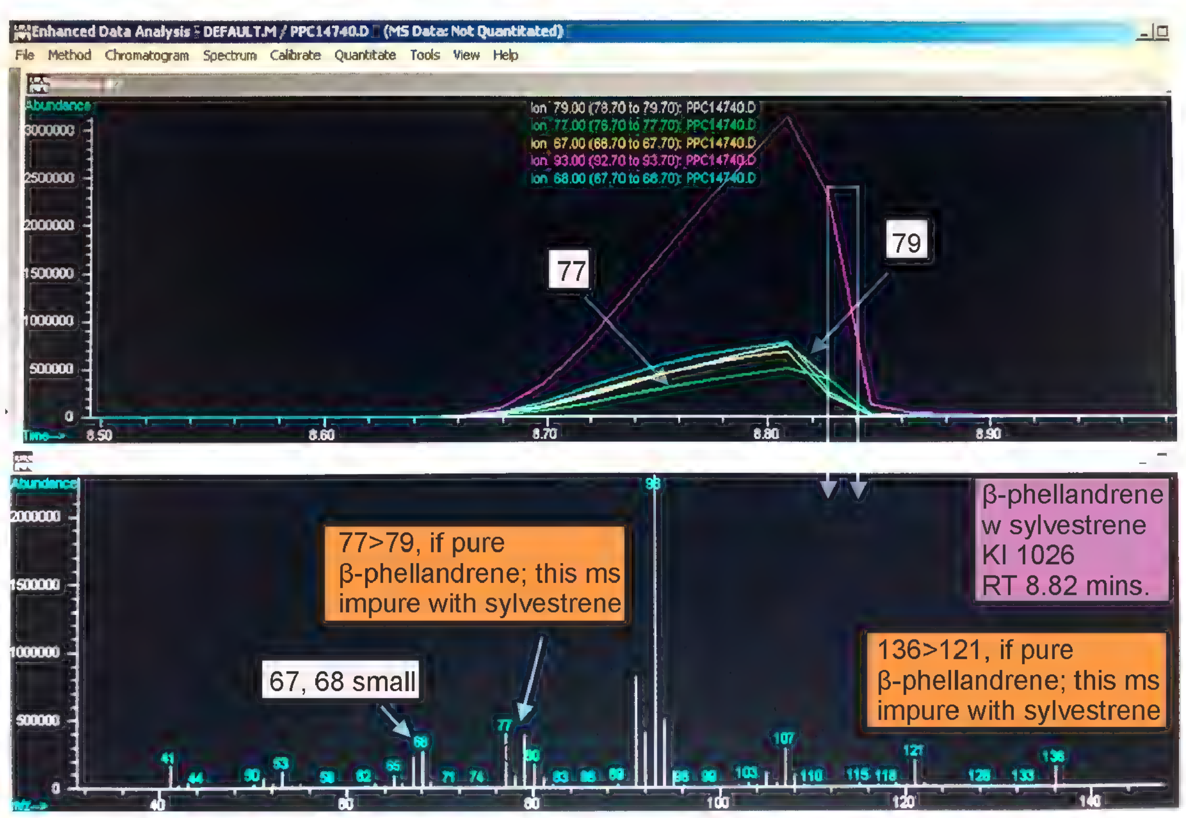


Fig. 2c. The MS of β-phellandrene (lower section) from the average of 8.828 to 8.846 mins in SIC region as shown by the 2 arrows (upper). The MS contains some sylvestrene contamination (cf. reference pure β-phellandrene MS in Fig. 1). Note the sharp decline in ion 67, signaling the end of the sylvestrene peak. β-phellandrene is about 5% of the SIC peak.

The amounts of limonene, sylvestrene and β-phellandrene were inconsistent (Table 1) and this probably reflects both the difficulty of identification and the lumping of compounds into one value in some reports. The Bulgaria oil consist of trace, 9.5 and 1.0% of limonene, sylvestrene and β-phellandrene (Table 1). Aside from the present report, sylvestrene has only reported by Ioannou et al. (2014) (Table 1). But, Karapandzova et al. (2011) list limonene + β-phellandrene as 2.0-6.6%, with no sylvestrene reported, so it seems likely their report is limonene + sylvestrene + β-phellandrene. Petrakis (Greece) reported only limonene (2.5%), with no sylvestrene or β-phellandrene, but because our Bulgaria plants are not far away from the Greece plant examined by Petrakis, and only one plant contained a trace amount of limonene, it seems likely that Petrakis (Greece) oil is mostly sylvestrene (~2.5%). Most studies found (1.0-6.8%) β-phellandrene, however, Petrakis and Ioannou reported no β-phellandrene in oils from Greece, nor from *P. peuce*, cultivated in Finland (Table 1). Clearly there is some confusion on the amounts of these compounds in some reports. Hopefully, the mass spectral methods presented in this paper will be of use to correct these reports in the future.

Generally, the Bulgarian oil composition is similar to that from other regions (Table 1). However, there are a few unusual amounts that are noted with ?? in table 1. Mitic et al. (2017) reported 1.1% β-gurjunene and 0.6% aromadendrene. Karapandzova et al. (2011) listed β-selinene as 0 to 0.4%. Petrakis et al. (2001) reported neryl acetate (0.9%), phenyl ethyl isovalerate (4.1%), and aristolene (0.6%), and these were not found in any other oils (Table 1). Koukos et al. (2000) reported 13.4% citronellol, and 0.0% germacrene D which are at odds with other reports.

VARIATION IN THE OIL COMPOSITION AMONG TREES IN BULGARIA

The major components were α-pinene (27.1-49/2%), camphene (2.8-8.6%), β-pinene (9.7-20.4%), myrcene (1.4-4.1%), sylvestrene (1.5-34.9%), β-phellandrene (0-1.4%), bornyl acetate (1.8-7.0%), (E)-caryophyllene (1.8-2.4%), germacrene D (8.7-10.6%) and germacrene-D-4-ol (0.3-1.1%).

The variation in sylvestrene was greatest with plants *14738* and *14740* having 34.9 and 12.5% in contrast to the other three plants that range from only 1.5-2.0%. This may constitute a sylvestrene chemotype (high and low) in *P. peuce*, but additional sampling is needed to confirm this.

ACKNOWLEDGEMENTS

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Table 1. The leaf oil of *Pinus peuce* from Bulgaria compared with published analyses. Major compounds in bold face yellow. Compositional values less than 0.1% are denoted as traces (t). ?? indicates a possible mis-identification in the literature. Unidentified components less than 0.5% are not reported. KI is the Kovat's Index using a linear calculation on DB-5 column.

KI	compound	Bulgaria this study *15775*	M-Serbia Mitic[1]	Serbia Nikolic[2]	Macedonia Karapand-zova[3]	Greece Petrakis[4]	Greece, Koukos[5]	cult Finland Ioannou[6]
921	tricyclene	0.4	-	0.7	t	-	0.4	0.5
924	α-thujene	-	0.6	t	t	0.4	-	-
932	**α-pinene**	**38.8**	**36.5**	**45.5**	**9.2-27.3**	**21.8**	**23.1**	**30.1**
946	camphene	6.1	8.5	10.0	0.9-6.4	2.5	5.5	5.9
948	benzaldehyde	-	-	-	t-1.4	-	-	-
967	(tert-butyl benzene)	-	-	-	0-7.8	-	-	-
969	**sabinene**	**t**	**0.7**	**t**	**0-0.3**	**0.3**	**-**	**-**
974	**β-pinene**	**16.2**	**6.8**	**10.8**	**6.0-13.1**	**9.4**	**22.0**	**10.8**
988	myrcene	2.7	1.0	0.9	t-1.4	4.0	2.0	1.5
1002	α-phellandrene	0.3	0.8	0.2	t-0.8	0.2	0.2	0.5
1008	**δ-3-carene**	**0.2**	**-**	t	0-1.7	0.1	0.5	0.1
1014	α-terpinene	t	-	t	0-1.2	0.2	0.3	0.1
1020	p-cymene	t	-	t	t-0.8	-	0.3	-
1024	limonene	t	-	-	?	2.5 ??	-	-
1025	**sylvestrene**	**9.5**	**??**	**??**	**??**	**??**	**??**	**4.7**
1025	**β-phellandrene**	**1.0**	**4.7**	**3.4**	**2.0-6.6 Lmn+βph**	**- ??**	**6.8**	**-**
1044	**(E)-β-ocimene**	**t**	**-**	t	0-0.3	0.7	-	t
1054	γ-terpinene	t	-	t	0-0.4	0.2	-	t
1065	cis-sabinene hydrate	t	-	-	-	-	-	0.3
1082	m-cymenene	-	-	-	-	-	-	-
1086	**terpinolene**	**0.5**	**0.5**	**0.3**	**0.3-0.7**	**0.6**	**- ??**	**0.9**
1095	linalool	-	-	-	-	t	-	-
1114	endo-fenchol	t	-	-	-	t	-	-
1118	cis-p-menth-2-en-1-ol	0.2	-	-	-	-	-	-
1122	α-campholenal	t	-	-	t-0.1	-	-	-
1135	trans-pinocarveol	-	-	t	0-0.3	-	-	-
1136	trans-p-menth-2-en-ol	0.2	-	-	-	-	-	-
1136	trans-sabinol	0	-	-	-	-	-	-
1141	camphor	0.3	-	-	0-0.2	-	-	-
1165	borneol	0.6	-	t	0-2.8	t	-	-
1174	terpinen-4-ol	t	-	t	0-0.1	0.1	-	0.1
1179	p-cymen-8-ol	t	-	-	0-0.1	-	-	-
1186	α-terpineol	0.2	-	-	0.1-0.4	0.5	-	-
1195	myrtenol	t	-	t	-	-	0.2	-
1195	cis-piperitol	t	-	-	-	-	-	-
1207	trans-piperitol	0.1	-	-	-	-	-	-
1215	trans-carveol	t	-	-	0-0.1	-	-	-
1218	endo-fenchyl acetate	t	-	t	0-0.1	0.3	-	-
1223	citronellol						**13.4 ??**	
1226	cis-carveol	t	-	-	-	-	-	-
1243	piperitone	t	-	-	t	-	-	-
1253	trans-sabinene hydrate acetate	-	-	0.1	-	-	-	-
1254	linalool acetate	t	-		t	-	0.2	-
1284	**bornyl acetate**	**4.5**	**6.8**	**4.5**	**0.2-11.7**	**- ??**	**9.8**	**6.4**
1342	trans-piperitol acetate	0.4	(1.6?)	0.8	-	-	-	-
1345	**α-terpinyl acetate**	**0.8**	**0.9**	**0.9**	**0-3.0**	**0.2**	**2.0**	**0.2**
1348	α-cubebene	-	-	-	-	-	-	0.4
1359	neryl acetate				-	**0.9 ??**	-	
1374	α-copaene	t	-	t	t-0.2	-	-	0.4
1379	geranyl acetate	-	-	-	t	0.4	-	-
1387	β-bourbonene	t	-	0.1	0.1-0.3	-	-	0.2
1389	β-elemene	t	-	0.2	0.1-1.3	-	-	0.6
1403	methyl eugenol	-	-	-	t	-	-	-
1407	longifolene	t	-	-	t	-	-	-
1409	α-gurjunene	-	-	-	-	1.0	-	-
1417	**(E)-caryophyllene**	**2.3**	**5.2**	**3.0**	**3.9-7.1**	**4.7**	**3.0**	**9.8**

KI	compound	Bulgaria this study *15775*	M-Serbia Mitic[1]	Serbia Nikolic[2]	Macedonia Karapand-zova[3]	Greece Petrakis[4]	Greece, Koukos[5]	cult Finland Ioannou[6]
1430	β-copaene	t	-	0.2	0.2-0.9	-	-	0.1
1431	β-gurjunene	-	**1.1 ??**	-	-	-	-	-
1439	aromadendrene	t	**0.6 ??**	-	t	-	-	-
1454	α-humulene	0.4	0.8	0.5	0.5-1.6	0.9	0.5	1.8
1454	(E)-β-farnesene	-	-	t	-	-	-	-
1478	γ-muurolene	-	0.6	-	0.1-1.2	-	-	t
1480	**germacrene D**	**9.7**	**11.4**	**11.1**	**7.1-19.9**	**18.8**	**- ??**	**17.0**
1489	β-selinene	t	-	-	0-0.4	-	-	-
1495	phenyl ethyl isovalerate				-	**4.1 ??**	-	
1500	bicyclogermacrene	0.6	-	0.6	0.6-2.0	-	-	1.2
1500	α-muurolene	0.1	1.2	t	0-1.9	1.0	0.3	0.2
1508	germacrene A	0.1	-	-	-	-	-	0.1
1513	γ-cadinene	0.2	0.5	-	0.4-2.7	-	0.4	t
1522	δ-cadinene	0.4	0.8	0.1	1.5-8.3	3.7	0.7	0.5
1537	α-cadinene	t	-	t	0.1-0.3	-	-	-
1561	(E)-nerolidol	-	-	-	t-3.1	-	-	-
1574	**germacrene-D-4-ol**	**0.6**	**0.5**	**0.9**	**0-3.7**	**-**	**-**	**t**
1583	caryophyllene oxide	0.1	-	t	0-0.7	-	-	-
1608	humulene epoxide II	t	-	-	0-0.4	-	-	-
1608	β-atlantol	t	-	-	-	-	-	-
1638	epi-α-cadinol	t	-	-	-	-	-	0.5
1640	epi-α-muurolol	t	-	-	-	-	-	-
1644	α-muurolol	-	-	-	-	-	-	0.5
1645	1-epi-cubenol	t	-	-	t-0.3	-	-	t
1652	α-cadinol	0.4	-	0.1	0.3-7.0	-	-	-
1685	germacra-4(15),5,10)14)-trien-1-al	t	-	0.2	-	-	-	-
1739	oplopanone	0.1	-	t	t	-	-	-
1759	benzyl benzoate	-	-	-	t	-	-	-
1762	aristolene	-			-	**0.6 ??**	-	
1959	hexadecanoic acid	0.2	-	-	-	-	-	-
1987	manool oxide	t	-	-	-	-	-	t
2014	palustradiene	t	-	-	-	-	-	-
2056	abietatriene	-	-	-	t	-	-	-
2087	abietadiene	-	-	-	t	-	-	-
2149	abienol	0.2	-	t	t	-	-	-
2220	isopimaral	t	-	-	-	-	-	-
2243	**palustral (8,13-abietadien-18-al**	**0.2**	-	-	-	-	-	-
2274	dehydro abietal	-	-	-	t	-	-	0.1
2310	isopimarol	-	0.0	-	-	-	-	-
2312	abieta-7,13-dien-3-one	-	-	-	-	-	-	-
2313	abietal	t	-	t	0-3.7	-	-	-

[1]Mitic et al. 2017; [2]Nicolic et al. 2014; [3]Karapandozova, et al. 2011; [4]Petrakis et al. 2001; [6]Ioannou et al. 2014; [5]Koukos et al. 2000.

Table 2. Variation in constituents of the leaf volatile oil of *P. peuce* within a population in Bulgaria. Major and diagnostic compounds are in bold face and yellow.

KI	compound	Bulgaria *14738*	Bulgaria *14740*	Bulgaria *14741*	Bulgaria *14737*	Bulgaria *14739*	Average *15775*
921	**tricyclene**	**0.2**	**0.5**	**0.5**	**0.5**	**0.2**	**0.4**
924	**α-thujene**	**-**	**-**	**-**	**-**	**-**	**-**
932	**α-pinene**	**27.1**	**31.4**	**36.4**	**47.4**	**49.2**	**38.8**
946	**camphene**	**2.8**	**7.9**	**8.6**	**8.1**	**2.9**	**6.1**
969	**sabinene**	t	t	t	t	t	**t**
974	**β-pinene**	**11.0**	**18.3**	**19.1**	**9.7**	**20.4**	**16.2**
988	myrcene	2.3	1.4	2.7	4.1	2.9	2.7
1002	α-phellandrene	0.4	0.4	0.3	0.3	0.2	0.3
1008	**δ-3-carene**	0.9	0.1	t	0.3	t	**0.2**
1014	α-terpinene	t	t	t	t	t	t
1020	p-cymene	t	t	t	t	t	t
1024	**limonene**	**-**	**t**	**-**	**-**	**-**	**t**
1025	**sylvestrene**	**34.9**	**12.5**	**2.0**	**1.9**	**1.5**	**9.5**
1025	**β-phellandrene**	**-**	**0.7**	**1.4**	**1.2**	**1.0**	**1.0**
1032	(Z)-β-ocimene	t	t	t	t	t	t
1044	(E)-β-ocimene	t	t	t	t	t	t
1054	γ-terpinene	t	t	t	t	t	t
1065	cis-sabinene hydrate	t	t	t	t	t	t
1086	**terpinolene**	**0.6**	**0.6**	**0.6**	**0.5**	**0.4**	**0.5**
1114	endo-fenchol	t	t	t	t	t	t
1118	cis-p-menth-2-en-1-ol	0.3	0.2	0.3	0.2	0.1	0.2
1122	α-campholenal	t	t	t	t	t	t
1136	trans-p-menth-2-en-ol	0.2	0.2	0.3	0.2	0.1	0.2
1141	camphor	t	0.5	0.5	0.4	0.2	0.3
1145	camphene hydrate	t	t	t	t	t	t
1158	trams-pinocamphone	t	t	t	t	t	t
1160	pinocarvone	t	t	t	t	t	t
1165	**borneol**	**t**	**1.0**	**0.8**	**0.7**	**0.3**	**0.6**
1174	terpinen-4-ol	t	t	t	t	t	t
1179	p-cymen-8-ol	t	t	t	t	t	t
1186	α-terpineol	0.4	0.4	0.2	t	0.2	0.2
1195	myrtenol	t	t	t	t	t	t
1195	cis-piperitol	t	t	t	t	t	t
1207	trans-piperitol	t	t	t	t	t	0.1
1215	trans-carveol	t	t	t	t	t	t
1218	endo-fenchyl acetate	t	t	t	t	t	t
1226	cis-carveol	t	t	t	t	t	t
1243	piperitone	t	t	t	t	t	t
1254	linalool acetate	t	t	-	t	t	t
1284	**bornyl acetate**	**2.2**	**5.5**	**7.0**	**5.5**	**1.8**	**4.5**
1342	trans-piperitol acetate	0.5	0.3	0.7	0.4	0.2	0.4
1345	**α-terpinyl acetate**	**0.7**	**0.7**	**1.1**	**0.9**	**0.6**	**0.8**
1374	α-copaene	t	t	t	t	t	t
1387	β-bourbonene	t	t	t	t	t	t
1389	β-elemene	t	t	t	t	t	t
1407	longifolene	t	t	t	t	t	t
1417	**(E)-caryophyllene**	**2.0**	**2.4**	**2.3**	**2.4**	**1.8**	**2.3**
1430	β-copaene	t	t	t	t	t	t
1439	aromadendrene	t	t	t	t	t	t
1454	α-humulene	0.3	0.4	0.4	0.4	0.3	0.4
1480	**germacrene D**	**9.2**	**8.9**	**10.6**	**9.6**	**8.7**	**9.7**
1489	β-selinene	t	t	t	t	t	t
1500	bicyclogermacrene	0.5	0.7	0.6	0.5	0.7	0.6
1500	α-muurolene	t	t	t	t	0.1	0.1
1508	germacrene A	t	0.2	t	t	0.1	0.1
1513	γ-cadinene	t	0.3	t	t	0.3	0.2
1522	δ-cadinene	0.3	0.6	0.33	0.3	0.5	0.4
1537	α-cadinene	t	t	t	t	t	t
1574	**germacrene-D-4-ol**	**0.3**	**1.1**	**0.4**	**0.3**	**1.0**	**0.6**
KI	compound	Bulgaria *14738*	Bulgaria *14740*	Bulgaria *14741*	Bulgaria *14737*	Bulgaria *14739*	Average *15775*

1583	caryophyllene oxide	t	0.1	t	0.1	0.1	0.1
1608	humulene epoxide II	t	t	t	t	t	t
1608	β-atlantol	t	t	t	t	t	t
1638	epi-α-cadinol	t	t	t	t	t	t
1640	epi-α-muurolol	t	t	t	t	t	t
1645	1-epi-cubenol	t	t	t	t	t	t
1652	α-cadinol	t	0.3	t	0.2	0.3	0.3
1685	germacra-4(15),5,10)14)-trien-1-al	t	t	t	t	t	t
1739	oplopanone	t	0.1	t	t	0.1	0.1
1959	hexadecanoic acid	0.2	0.2	t	t	0.2	0.2
1987	manool oxide	t	t	t	t	t	t
1987	pimara-7,15-diene<iso->	t	t	t	t	t	t
2014	palustradiene	t	t	t	t	t	t
2149	abienol	t	t	0.3	0.2	0.4	0.2
2220	isopimaral	t	t	t	t	0.1	t
2243	**palustral (8,13-abietadien-18-al**	**0.2**	**0.2**	**0.1**	**0.2**	**0.2**	**0.2**
2297	methyl isopimarate	t	t	t	t	t	t
2313	abietal	t	t	t	t	t	t

Trifolium albopurpureum **var.** ***columbinum*** **(Fabaceae), a New Combination for a California Clover**

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ABSTRACT

Trifolium columbinum Greene is the correct name at the species level for the taxon that has been incorrectly called *T. olivaceum* Greene in various California floras. ***Trifolium albopurpureum*** Torr. & A.Gray var. ***columbinum*** (Greene) D.J.Keil (Fabaceae), **comb. nov.** is proposed to replace the illegitimate *Trifolium albopurpureum* var. *olivaceum* (Greene) Isely. *Phytologia 101(2): 131-133 (June 21, 2019).* ISSN 030319430.

KEY WORDS: Fabaceae, *Trifolium*

Greene (1887) proposed nine new species of *Trifolium* L. (Fabaceae). Jepson (1901) treated two of these, *Trifolium columbinum* Greene and *T. olivaceum* Greene as conspecific, creating the combination *T. columbinum* var. *olivaceum* (Greene) Jeps. for the latter. McDermott (1910) also treated *T. columbinum* and *T. olivaceum* as conspecific, but combined them under the latter name as *T. olivaceum*, creating the combination *T. olivaceum* f. *columbinum*. Jepson (1911) followed McDermott's classification. In a subsequent publication, Jepson (1925), while again treating these taxa as conspecific, published the combination *T. olivaceum* var. *columbinum* and used this combination again in a later publication (Jepson 1936). Munz (1959), following Jepson (1925, 1936), accepted *T. olivaceum* for the combined species. Hoover (1970) pointed out this error: "Jepson in 1901 first included *T. columbinum* and *T. olivaceum* Greene in one species under the former name. The subsequent use of the name *T. olivaceum* for the inclusive species is therefore incorrect." However, the incorrect use of *T. olivaceum* persisted, with Vincent and Isely (2012a) recognizing *T. olivaceum* at the species level for the taxon including *T. olivaceum* and *T. columbinum*.

According to Article 11.5 of the *International Code of Nomenclature for algae, fungi, and plants* (Turland et al. 2018): "When, for any taxon at the rank of family or below, a choice is possible between legitimate names of equal priority at the corresponding rank, or between available final epithets of names of equal priority at the corresponding rank, the first such choice to be effectively published (Art. 29–31) establishes the priority of the chosen name, and of any legitimate combination with the same type and final epithet at that rank, over the other competing name(s)." As was pointed out by Hoover (1970), the correct name at the species level for a taxon that merges *T. columbinum* and *T. olivaceum* is *T. columbinum*, and in January 2019 this correction was incorporated into the Jepson eFlora (Vincent and Isely 2012b; T. Rosatti, pers. comm.), and it will be used in the upcoming treatment of *Trifolium* for *The Flora of North America North of Mexico* (M. Vincent, pers. comm.).

I am nearing completion of the second edition of *Vascular Plants of San Luis Obispo County, California* (Keil and Hoover, in prep.). Hoover (1970) pointed out in the first edition that "Many of our plants have corollas intermediate in size between typical *T. columbinum* and *T. albopurpureum*. This form has been called *T. olivaceum* var. *griseum* Jepson." I have examined many specimens of this complex, and I concur. Isely (1980) recognized this intermediacy and published the combination *Trifolium albopurpureum* Torr. & A.Gray var. *olivaceum* (Greene) Isely, and used this combination in subsequent publications (Isely 1993, 1998). However, Isely was apparently unaware that the autonym

columbinum has priority at the varietal level (ICN Article 11.6—Turland et al. 2018), and therefore the combination *T. albopurpureum* var. *olivaceum* is illegitimate.

Zohary and Heller (1984) went a step further and wholly merged these taxa, recognizing *Trifolium albopurpureum* sensu lato, including as synonyms various entities that previous workers had recognized as species, varieties, and formae. Although these authors had a broad perspective of the genus *Trifolium*, they disregarded patterns of variation among members of the *T. albopurpureum* complex that most California botanists have recognized. In my opinion merging all the variants under a single species name without recognition of infraspecific variants masks important patterns of ecogeographical variation, at least some of which may be evolutionarily important.

I agree with Isely that *T. columbinum* is better treated as a variety of *Trifolium albopurpureum* than as a separate species, and I plan to so treat these plants in *Vascular Plants of San Luis Obispo County, California*. However, because a legitimate combination in *T. albopurpureum* is unavailable, I propose the following new combination:

Trifolium albopurpureum Torr. & A.Gray var. ***columbinum*** (Greene) D.J.Keil, *comb. nov.* —*Trifolium columbinum* Greene, Pittonia 1: 4. 1887; *Trifolium olivaceum* Greene f. *columbinum* McDermott, Ill. Key Amer. Trifolium 213, pl. 86. 1910, *nom. illeg.*; *Trifolium olivaceum* Greene var. *columbinum* (Greene) Jeps., Man. Fl. Pl. Calif. [Jepson] 546. 1925, *nom. illeg.* Type: USA. California. [Solano Co.]: Vacaville, 2 May 1886, *E. L. Greene s.n.* (holotype: ND-G 37926 [barcode NDG67001], image!; isotypes: CAS (2), images!; NY, image; PH, image!

Trifolium olivaceum Greene, Pittonia 1: 4. 1887; *Trifolium columbinum* Greene var. *olivaceum* (Greene) Jeps., Fl. W. Calif. [Jepson] 307. 1901; *Trifolium albopurpureum* Torr. & A.Gray var. *olivaceum* (Greene) Isely, Brittonia 32: 55. 1980, nom. illeg. Type: USA. California. [Solano Co.]: Vacaville, 3 May 1886, *E. L. Greene s.n.* (holotype: ND-G 37925 [barcode NDG67266], image!; isotype: CAS, image!).

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Inheritance of chloroplasts and mitochondria in Conifers: A review of paternal, maternal, leakage and facultative inheritance

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ABSTRACT

The inheritance of chloroplasts and mitochondria in conifers is reviewed. Generally, in the Pinaceae, chloroplasts are paternally inherited and mitochondria maternally inherited. In contrast, most other conifers have paternal inheritance for both chloroplasts and mitochondria. Several examples of leakage are discussed where normally paternal cp or mt are inherited by 1-3% maternal leakage. A few *Pinus* taxa with mt maternal, have leakage of 1-5% paternal. An unusual case of facultative inheritance is discussed where both forward and reciprocal crosses of *Pinus mugo* x *P. sylvestris* resulted in hybrids with *P. mugo* chloroplasts. Published on-line www.phytologia.org *Phytologia 101(2):134-138 (March 21, 2019).* ISSN 030319430.

KEY WORDS: inheritance, chloroplasts, mitochondria, conifers

It is estimated that in angiosperms, chloroplasts (cp) are maternally inherited in over 70% of the genera (Hipkins et al. 1994) and about 25% of genera are biparentally inherited (Harris and Ingram (1991). Interestingly, chloroplasts were found to be only maternally inherited in dicot trees according to Rojara and Dancik (1992). However, in conifers, chloroplasts and mitochondria (mt) exhibit considerable variation in their inheritance (Table 1).

In the Pinaceae, chloroplasts are generally inherited paternally (via pollen) and mitochondria generally maternally inherited (Table 1). However, maternal and paternal leakage has often been reported Table 1). Maternal leakage refers to persistence of a few (or sometimes many) chloroplasts and/or mitochondria from the female egg, due to the incomplete degradation during fertilization (Mogensen, 1996). Likewise, paternal leakage is the survival of a few (or many) chloroplasts and/ or mitochondria from the pollen during fertilization (Mogensen, 1996). Mogensen (1996, figs. 24-29, etc.), in his seminal paper, gives lucid details for mechanisms for the destruction of cell organelles and he writes several times how "this mechanism could lead to some 'leakiness' of maternal plastids", "resulting in proembryo cells **typically** (emphasis added) containing **only** male cytoplasm", and "in the Pinaceae, the mechanism of cytoplasmic transmission described above could lead to some leakiness".

Scmidt et al. (1987) reported leakiness in *Larix decidua* x *leptolepis* in which 5 hybrids exhibited paternal inheritance, but one of the 6 hybrids had maternal chloroplast DNA, and 2 other hybrids had mixed Bam-HI restriction patterns indicative of recombination between paternal and maternal cp DNAs.

Analyses of mitochondria DNA RFLPs of 125 hybrid seedlings originating from 23 controlled crosses of *Pinus banksiana* x *P. contorta* revealed 119 maternal and 6 paternal mitochondria (~5%, Wagner, et al. 1991). Cato and Richardson (1996) utilized cpSSRs to examine inheritance of chloroplast in 206 progeny obtained from controlled pollinations of 4 *Pinus radiata* families. The found 99% of the progeny inherited their chloroplast paternally (pollen). But, approximately 1% of the progeny contained maternal chloroplasts, thus showing leakage in chloroplast inheritance.

Owens and Morris (1991) studied cytoplasmic inheritance in *Pseudotsuga menziesii* and noted that "most of the proembryo cytoplasm is of paternal origin, but some maternal organelles may be included". They estimated that about 10% of the final number of mitochondria were carried to the proembryo, along with paternal organelles.

Recently, Kormutak et al. (2017, 2018) have reported a most unusual inheritance of chloroplasts in reciprocal crosses of *Pinus mugo* and *P. sylvestris*. They found *P. sylvestris* (female) x *P. mugo* (male) yielded the typical *Pinus* inheritance of paternal (*P. mugo*) chloroplasts in the hybrids. However, *P. sylvestris* (male) x *P. mugo* (female) resulted in all hybrids with *P. mugo* chloroplast DNA (maternally inherited!). If this case can be generalized to natural hybridization of *P. sylvestris* and *P. mugo*, then the analysis of hybrids with only *P. mugo* chloroplasts will likely make studies of hybridization and introgression between these species very difficult. In any case, this example does offer a novel mechanism for chloroplast capture.

The phylogenetic distinctiveness of the Pinaceae is demonstrated by the shift in mode from cp (paternal), mt (maternal) in the Pinaceae to cp (paternal), mt (paternal) in other conifer families (Table 1), although the Taxaceae (*Taxus bacata*) may have cp (paternal), mt (maternal), but the literature is unclear as Mogensen (1996) cites Pennell and Bell (1988) who concluded "There is the **possibility** (emphasis added) that, as in some other gymnosperms, the plastids and mitochondria in the zygote come **in part** from the male gametophyte".

Cunninghamia konishii (Cupressaceae) seems to be an exception to all conifers Lu, et al. 2001) in having maternal inherited chloroplasts (Table 1), with the exception of the unusual case of *P. mugo* x *P. sylvestris* (above).

As in the case with the *Pinaceae*, maternal leakage has been reported in other conifer families (Table 1). Ohba et al. (1971) examined the inheritance of the Wogon-Sugi trait (i.e., plants having white-yellowish leaf tips, a chlorophyll mutation) in *Cryptomeria japonica.* They noted "for Wogon-Sugi (,) the trait was **mostly** (emphasis added) transmitted to the progeny when Wogon-Sugi (plant) was used as pollen parent". So, apparently, some maternal leakage occurs in *Cryptomeria japonica.*

Recently, Kou et al. (2014) re-examined the classical intergeneric hybrid, Leyland cypress (*Chamaecyparis nootkatensis* x *Cupressus macrocarpa*, now *Callitropsis nootkatensis* x *Hesperocyparis macrocarpa*). Keith Rushforth and I provided historical information on the maternal seed sources of the Leyland cypress analyzed in the study (Tables 2, 3, Kou et al. 2014). Six Leyland cypress cultivars, and putative parents or exemplars were analyzed using sequences from 3 mt genes (*cox*I, *atp*A, and *rps*3) and 2 cp genes (*mat*K and *rbc*L). Four cultivars from seed of *H. macrocarpa* trees (pollen ex *C. nootkatensis*) had *C. nootkatensis* type chloroplasts and mitochondria. However, two cultivars whose seed purportedly came from a *C. nootkatensis* (maternal) tree (x pollen of *H. macrocarpa*), were grown and contained 2 unusual seedlings: Green Spire and Haggerston Grey. These 2 cultivars were found to have *H. macrocarpa* type chloroplasts and mitochondria. Thus, if the historical records on seed source are correct, the six Leyland cypress cultivars contained 4 showing paternal inheritance and 2 exhibited maternal inheritance. Unfortunately, these results rest on historical records of plant growth and plantings, which are, of course, subject to error.

Another Cupressoideae taxon, *Chamaecyparis obtusa*, was studied (Shiraishi et al. 2001) by use of a cpDNA region, trnD-trnY. In 361 progenies. 352 (97.5%) had the same haplotype as their male parent (paternal) and 9 (2.5%) had the haplotype of their female parent (maternal), showing that although chloroplasts are mostly inherited paternally in *Chamaecyparis obtusa*, there is maternal leakage.

Table 1. Inheritance of cp (chloroplasts) and mt (mitochondria) in conifers. ns = not studied.

	cp	mt	ref.
Pinaceae	pat	mat	review, Mogensen, 1996.
Larix decidua x *leptolepis*	pat[1]	mat	DeVerno et al. 1991; Scmidt et al. 1987
	[1] 1 hybrid had mat cp (i.e., leakage)		
Picea abies	ns	mat	Grivet, et al. 1999
Picea sp.	pat	mat	Sutton et al. 1991
Pinus sp.	pat	mat	Wagner et al. 1987; Neale and Sederoff, 1988, 1989
Pinus banksiana x *contorta*	ns	mat[2]	Wagner et al. 1991
		[2] ~5% pat leakage	
Pinus echinata, Pinus elliotii, Pinus palustris, and *Pinus taeda*	pat	ns	Wagner et al. 1992
Pinus radiata	pat[3]	ns	Cato and Richardson, 1996
	[3]~1% mat leakage		
Pinus sylvestris(female) x *P. mugo* (male)	pat	ns	Kormutak, et al. 2017, 2018
Pinus mugo (female) x *P. sylvestris* (male)	mat	ns	Kormutak, et al. 2017, 2018
Pinus taeda	pat	mat	Neal and Sederoff, 1988, 1989
Pseudotsuga menzesii	pat[4]	mat[4]	Marshall and Neale, 1991; Wagner et al. 1989
[4]some organelles from mat(cp) or pat(mt) may be included.			Owens and Morris, 1991
Taxaceae			
Taxus bacatta	pat?	mat?	Pennell and Bell, 1988, Mogensen, 1996.
Araucariaceae			
Agathis robusta	pat	pat	Kaur and Bhatnager, 1984
Cephalotaxaceae			
Cephalotaxus drupacea	pat	pat	Gianordioli, 1974; Singh, 1961
Cupressaceae			
Cunninghamioideae			
Cunninghamia konshii	mat	ns	Lu, et al. 2001
Sequoioideae			
Sequoia sempervirens	pat	pat	Neale, Marshall and Sederoff, 1989
Taxodioideae			
Cryptomeria japonica	pat[5]	ns	Ohba et al. 1971
	[5]some mat leakage		
Callitroideae			
Callitris (4 species)	pat	ns	Sakaguchi, et al. 2014
Cupressoideae			
Leyland cypress - *Callitropsis nootkatensis* (= *Chamaecyparis nootkatensis*) x *Hesperocyparis* (= *Cupressus*) *macrocarpa* 4 plants:	pat	pat	Kou, et al. 2014
2 plants:	mat	mat	Kou, et al. 2014
Calocedrus decurrens	pat	pat	Neale, Marshall and Harry, 1991
Chamaecyparis obtusa	pat[6]	ns	Shirashi et al. 2001
	[6]~2.5% mat leakage		
Chamaecyparis obtusa x pisifera	pat	pat	Kondo, et al., 1998
Chamaecyparis lawsonia	pat	pat	Chesnoy, 1973
Platycladus orientalis	pat	pat	Chesnoy, 1969
Hesperocyparis arizonica x *H. macrocarpa*	pat	ns	Adams et al. 2018
Juniperus ashei, J. pinchotii, J. virginiana	pollen	pollen	Mohanty et al. 2016, ultrastructural presence of cp and mt in pollen was confirmed by TEM and DNA.

Mohanty et al. (2016) examined the pollen of *Juniperus ashei*, *J. pinchotii* and *J. virginiana* by TEM (Transmission Electron Microscopy) and verified the presence of chloroplasts and mitochondria in pollen in these three species. They also perfected a method to extract DNA from single pollen grains and verified specific cpDNA markers that distinguished these three species. Unfortunately, they did not have access to hybrids from controlled crossings of junipers, so they could not comment on the inheritance of chloroplasts and mitochondria in *Juniperus*.

However, Scion Ltd., New Zealand has made controlled crossings in a closely related genus, *Hesperocyparis*. Adams et al. (2018) recently analyzed 18 hybrids from a single, controlled cross, *H. arizonica* (male) x *H. macrocarpa* (female), and all 18 had perfect *H. arizonica* (paternal) chloroplast DNAs, confirming paternal inheritance of chloroplasts in *Hesperocyparis* (Table 1).

In conclusion, from this brief review of the inheritance of chloroplasts and mitochondria in conifers, it seems very apparent that the literature is divided by the earlier, genetics works (mostly before 1996) and the newer, more molecular reports (1996- present), that will superseded by NextGen technologies, sequencing individual DNA stands, that will very likely reveal unusual modes of variation. We will surely discover more unusual mechanisms of chloroplast and mitochondria inheritance similar to the case of facultative selection reported by Kormutak et al., (2017, 2018).

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Taxonomic summary of *Rhizoclosmatium* and description of four new *Rhizoclosmatium* species (Chytriomycetaceae, Chytridiales)

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ABSTRACT

Rhizoclosmatium globosum, the type for the genus *Rhizoclosmatium*, is commonly isolated from freshwater aquatic systems, especially on chitin-containing substrates. The genus *Rhizoclosmatium* currently includes three additional described species: *R. aurantiacum* and *R. hyalinum*, which can grow on chitin, and *R. marinum* reported growing on a marine green alga. Our study employed analyses of ribosomal gene sequences, zoospore ultrastructure, and thallus morphology and development to identify chytrid strains related to *Rhizoclosmatium*. Phylogenetic analyses of ribosomal genes of strains revealed three major lineages within a clade that includes the type*, R. globosum*. Based on these results, we describe four new species and one new variety of *Rhizoclosmatium,* all capable of growth on chitin: *R. sparsum, R. umbonatum, R. umbonatum* var. *sphaericum, R. persicum,* and *R. pessaminum*. We have also found that *R. globosum* strains can grow on a range of substrates, including chitin, cellulose, keratin, and pollen. This information is herein assimilated into the existing taxonomy of the genus, which is also summarized and nomenclaturally updated. Published on-line www.phytologia.org *Phytologia 101(2): 139-163 (June 21, 2019).* ISSN 030319430.

KEY WORDS: *Rhizoclosmatium sparsum, R. persicum, R. pessaminum, R. umbonatum,* cellulose, chitin, chytrid, Chytridiomycota**,** keratin, morphology, phylogeny, pollen, systematics, ultrastructure, zoospore.

Chitin is the second most abundant organic compound in nature (Keyhani and Roseman, 1999), and understanding its turnover is critical to understanding parts of the carbon and nitrogen cycling in aquatic ecosystems (Gooday, 1990). Turnover of chitin polysaccharides in marine water columns and sediments is attributed primarily to bacteria (Gooday, 1990; Keyhani and Roseman, 1999). The identity of the microbes degrading chitin in freshwater systems is less well studied, but it is known that it includes fungi, particularly chytrid fungi (Gooday, 1990; Sparrow, 1960). The role of chytrids in aquatic food webs and in nutrient recycling has recently been emphasized (Kagami et al., 2007a, 2007b, 2011, 2014, 2017; Rasconi et al., 2014). Thus, knowledge of the biodiversity of chitinophilic chytrids is fundamental to understanding nutrient cycling in aquatic systems.

In an early study of aquatic fungi on insect exuviae, Petersen (1903) highlighted chitin-containing materials as substrates for chytrids. In his study of inhabitants of insect exuviae, he erected the genera *Rhizoclosmatium* and *Siphonaria* and observed *Obelidium*. Interestingly molecular phylogenetic analysis of a broad range of chytrid taxa (James et al., 2006) revealed that *Rhizoclosmatium globosum*, the type of the genus, forms a distinct clade with four other genera (*Obelidium, Siphonaria, Podochytrium* and *Phlyctorhiza)*, all of which utilize chitin as a substrate. *Rhizoclosmatium globosum* is one of the most commonly reported chytrids isolated from freshwater aquatic habitats (Sparrow, 1960). Phylotypes of *Rhizoclosmatium* sp. have even been detected unexpectedly in municipal drinking water (Otterholt and Charnock, 2011). Its prevalence has been demonstrated from complementary isolation, culture, and

environmental sequence studies (Davis et al., 2013, 2016, 2018; Lefèvre et al., 2012). These studies also revealed molecular divergence of strains with morphologies somewhat similar to *R. aurantiacum*, suggesting greater species diversity than currently recognized (Davis et al., 2016) and highlighting the need for greater study of the genus.

Herein we summarize the taxonomy of the genus *Rhizoclosmatium* and explore the relationships of strains in culture, including *R. globosum* and unidentified species that can be placed in this genus based on molecular phylogenetics and morphology. In addition to morphology, we present zoospore ultrastructure and analyses of ribosomal gene sequences. From these analyses we establish four additional species and one variety in this genus.

MATERIALS AND METHODS

Culture and morphological observations: Collecting sites and isolation baits for strains included in this study are listed in Table 1. Strains brought into pure culture were maintained on PmTG agar (1 g peptonized milk, 1 g tryptone, 5 g glucose, 10 g agar, 1 L distilled water). After growth for seven days on PmTG agar, strains were flooded with sterile distilled water for 15-30 min., which resulted in discharge of zoospores. We inoculated PmTG agar and natural substrates (sweet gum pollen, chitin from shrimp exoskeletons, cellulose strips from onion bulb scale epidermis, and keratin from molted snake skin) with zoospore suspensions, incubated at 22° C, and made light microscopic observations of strains over a 1-5 day period using either a Nikon Eclipse E200 or Zeiss Axioskop microscope equipped with bright field, phase contrast and Nomarski interference contrast optics.

DNA extraction, purification, amplification and sequence alignment: After growing strains for 3–5 days in PmTG broth on a rotary shaker, we extracted and purified genomic DNA as previously described (Davis et al., 2013). Complete ITS1-5.8S-ITS2 (ITS) and partial large subunit RNA (LSU; 28S rDNA) genes were amplified with primer pairs: ITS5/ITS4 and LROR/LR5 respectively (Rehner and Samuels, 1994; Vilgalys and Hester 1990; White et al., 1990). Following PCR reactions (Davis et al., 2013), amplicons were purified with a Nucleospin Extraction II kit (Macherey-Nagel, Inc., Bethlehem, PA) and sequenced by Macrogen USA (Rockville, MD). We assembled the resulting sequences with Sequencher 4.5 (Genecodes) as previously described (Vélez et al., 2011). Our study includes 16 newly recorded 28S rDNA sequences for strains (Table 1) and eight new ITS sequences for the following strains: JA 20=MK314726; MB 07=MK314722; MB 10=MK314723; MB 48=MK314727; MP 49=MK314724; MP 73=MK314728; WB 219=MK314725; WB 236B=MK314729.

Molecular phylogenetic analyses: We used newly generated 28S rDNA sequences along with sequences downloaded from GenBank as shown in Table 1. Sequences were aligned with Clustal X (Thompson et al., 1997) and manually adjusted in BioEdit (Hall, 1999). Maximum parsimony (MP) trees were generated using PAUPRat (Sikes and Lewis, 2001), and bootstrap support values were generated from heuristic searches with 500 replicates, each with 10 random-stepwise addition replicates. Maximum likelihood (ML) phylogenies were constructed using GARLI 0.951 (Zwikl, 2006) as explained in Vélez et al. (2011). The best model of base substitution was selected using Modeltest 3.7 (Posada and Crandall, 1998). The best tree was obtained from 100 best tree searches using the HKY model of nucleotide substitution. Bootstrap values were calculated using 500 nonparametric replicates with the same substitution model. Trees were rooted with strain BR 097, *Chytriomyces hyalinus* (Vélez et al., 2011 show species identification of BR 097 is not *Chytriomyces (Chytridium) confervae*). Sequence similarities among strains of interest were calculated by pairwise alignment in BioEdit.

Zoospore ultrastructure: Zoospore ultrastructural organization of the following strains was examined: ATCC 22918, MP 44, MP 67, WJD 111, and WJD 185. Zoospore suspensions were fixed with 2.5% glutaraldehyde in 0.1 M sym-collidine buffer for 1 hr. at 21° C, washed three times in 0.1 M buffer, and

secondarily fixed with 1.0% osmium tetroxide in 0.1 M buffer for 1 hr. at 21° C in the dark. Following three washes with deionized water, zoospores were centrifuged at ~ 3× *g* and then infused with molten agar. After solidification, material was cut into blocks (~ 0.1–0.2 cm^3) and stained overnight in saturated aqueous uranyl acetate at 5° C. Material was then processed for electron microscopy as previously described (Powell et al., 2013). Stained sections were observed at 60 kV on a Hitachi 7650 transmission electron microscope (TEM).

RESULTS

Phylogenetic analysis: The dataset had 822 characters, of which 103 were parsimony informative. Our MP (L = 177 steps) and ML (-lnL = 693.51) phylogenetic assessments were identical, and the MP tree is presented (Fig. 1). In the MP tree, strains identified based on morphology as *Rhizoclosmatium* species place into three major well-supported lineages (Fig. 1, lineages A, B, C).

Lineage A with ≥99% support includes a clade (A1) of 29 *Rhizoclosmatium globosum* strains (≥99% support) and a sister clade (A2) of four strains (≥92% support) morphologically distinct from *R. globosum* and other described *Rhizoclosmatium* species. Cultures of strains in lineage A range in color from cream, light tan to rose-white. Within the *R. globosum* clade (A1), 28S rDNA gene sequence similarity ranges from 99.8-100%; ITS gene sequence similarity ranges from 99.3-100%. For example, the ITS sequence in strain JEL 347h (AY997076) is 100% similar to that of strain MB 49 (MK314724); 99.6% similar to that of strains WB 236B (MK314729) and MP 73 (MK314728); and 99.3% similar to that of strain MB10 (MK314723).

Divergence in the 28S rDNA and ITS genes distinguishes the two sister clades in Lineage A, justifying a new species. There is 98% 28S rDNA sequence similarity between strain JEL 347h (A1) and strain MP 56 (A2), and only 91.2% ITS sequence similarity between strain JEL 347h (*R. globosum* AY997076) and strain MP 56 (JX905553). In contrast within clade A2, there is greater ITS similarity, 99.8% similarity between strain MP 56 (JX905553) and strain MP 46 (JX905552) and 100% sequence similarity with strain WB 266C (JX905558). This is a range of ITS differences similar to that found in the *R. globosum* clade (A1).

Lineage B with ≥81% support (Fig. 1) is sister of lineage A and includes five strains in two clades (B1, B2). Cultures of strains in lineage B range in color from bright orange (JEL 128) to light orange (MP 44). Strains in lineage B have 95% 28S rDNA sequence similarity with JEL 347h *R. globosum* of lineage A. Thalli in the two clades of lineage B share the feature of an umbo on developing thalli from the time of zoospore encystment to zoospore discharge. Lineage B strains MP 44 (B1) and JEL 128 (B2) are 99.25% similar in their 28S rDNA sequences, but thallus differences support the two clades as varieties of a new species, one from Alabama (B1) and the other from Maine (B2).

Lineage C with ≥90% support (Fig. 1) consists of two well-supported clades (C1, C2), each with 100% support, and is sister of lineages A + B. Lineage C is molecularly divergent from lineage A; the 28S rDNA sequence in strain MP 67 (C1) is 89.3% similar to that of JEL 347h (A1). Cultures of strains in lineage C are orange. Five strains of a new species are in a clade (C1) sister of two strains of another new species (C2). Strain MP 67 (C1) is 93.3 % similar in its 28S rDNA sequence to that of the two strains in the sister clade (C2=JEL 823, JEL 849). Morphology and molecular sequence differences support two new species within lineage C.

Zoospore ultrastructure: The zoospore is elongate (Fig. 2A) and exhibits features consistent with a Group 1-type zoospore (Barr, 1980; Barr and Hartman, 1976). A cell coat covers the body of the zoospore (Fig. 2G) but not the flagellum. A thin, biconcave flagellar plug lies in the transition region of the axoneme (Fig. 2A). A veil is adjacent to the non-flagellated centriole (Fig. 2D). The kinetosome-

associated structure (KAS) consists of two sets of stacked plates, each at the side of the kinetosome (Fig. 2C, D). Microtubules are bundled as a microtubule root, which extends from the side of the kinetosome and passes between the KAS plates toward the microbody-lipid globule (MLC) fenestrated cisterna (Fig. 2I). Stacked cisternae of the Golgi apparatus are in the posterior end of the zoospore near the microtubular root, and one cisterna is partitioned (Fig. 2H).

The nucleus is partially inserted into the ribosomal aggregation (Fig. 2A, B) in the orange-pigmented *R. persicum* (strain MP 67) and *R. umbonatum* (strain WJD 185), but could be partially inserted or outside the ribosomal aggregation in zoospores of the hyaline to pink strain *R. globosum* (WJD 111). The MLC typically includes a single lipid globule, adjacent microbody and fenestrated membrane cisterna, and proximal mitochondria (Fig. 2C, E, F, J). Mitochondria are positioned between the binding ER and the ribosomal aggregation (Fig. 2A). A large paracrystalline inclusion (Fig. 2K, L) lies in the peripheral cytoplasm (see Letcher and Powell 2014 for definition).

Morphology and Habitat: Strains differ in color in culture, and the pigmentation can change with the age of the culture. Strains in lineage A are lighter, pinkish white to light tan. Strains in lineage B are darker than those in lineage A, and are light orange (MP 44, WJD 185) to dark orange (JEL 128, JEL 796). Strains in lineage C are bright orange. Although all strains can grow on chitin, strains can use a variety of substrates in addition to chitin, including cellulose, keratin, and pollen (Table 1). Strains of *Rhizoclosmatium* were found in a range of aquatic habitats including ponds, bogs, fens, vernal pools, lakes and rivers often in samples containing aquatic or bog vegetation (Table 1).

TAXONOMY

Rhizoclosmatium currently includes four described species, three as saprotrophs on pollen and chitin-containing substrates and one as a marine algal parasite (Sparrow, 1960). Herein we expand the number of species in *Rhizoclosmatium* and confirm shared morphological features for the genus. The thallus is monocentric, with globose to subglobose sporangia, and a rhizoidal system emerges from a subsporangial apophysis that is variously shaped (Figs. 3B; 4G, I; 5H, I; 6I; 7B, C, F; 8D). Tips of rhizoids are pointed (Fig. 8D, G), and a domed-shaped septum separates the rhizoidal system from the sporangium (Figs. 4K; 7F; 8G, H). During development of the incipient sporangium from the encysted zoospore, the primary nucleus (Antikajian, 1949) enlarges before mitosis takes place (Figs. 4I; 5K; 7B) and divides only after the sporangium has achieved its mature size. Zoospores escape through a single inoperculate discharge pore (Figs. 4K; 5M; 7D, E, F) or papilla into a vesicle where they swarm as a mass before swimming away (Figs. 3H; 4J; 5M; 7E; 8F, G). The position of the discharge pore varies from apical, subapical, lateral (Figs. 3F, G; 4K; 5L, M; 6J; 7D, E, F; 8G) to basal. Sparrow (1937) illustrates and describes basal discharge in *R. aurantiacum,* but we did not observe basal discharge in any of our orange strains in culture. Consequently, we do not consider any of our orange strains as *R. aurantiacum*. Zoospores are posteriorly uniflagellate and vary in shape from elongate, oval to spherical (Figs. 3A; 4A; 5A; 6A; 8A). Resting spores when present are spherical and epibiotic. Sparrow (1937) reported sexual reproduction by rhizoidal conjugation of contributing thalli, but we did not observe rhizoidal conjugation in any of the strains studied. Much variation in rhizoidal complexity and sporangial and apophysis shape is found in these species. Below we summarize the current species, describe four new species and one variety, and provide a key to species of *Rhizoclosmatium*.

Rhizoclosmatium H. E. Petersen, Journ. de Botanique 17:216, 1903.

Mycobank MB 20480

Typification: Rhizoclosmatium globosum H. E. Petersen 1903 (TYPE SPECIES)

Description: Thallus consists of a spherical to variably shaped sporangium, with a single basal, subapical or apical inoperculate discharge pore and rhizoids that arise from a subsporangial swelling, the apophysis. Rhizoids fine or coarse tapering to pointed tips. Zoospores are fully cleaved within

sporangium and are released into an evanescent vesicle in which they swarm before swimming away. Zoospores are posteriorly uniflagellate. Primary nucleus in incipient sporangium enlarges, undergoing mitosis when the sporangium is fully expanded.

Rhizoclosmatium globosum H. E. Petersen, Journ. de Botanique 17: 216, FIGS. 1, 2, 1903.
= *Phlyctochytrium powhatanensis* Roane; Mycologia 65: 535, 1973.
Mycobank MB 225931 Fig. 3
Typification: DENMARK. Séeland, from water on insect exuviae, **LECTOTYPE designated here,** MBT385827, FIG. 1 in H. E. Petersen. Journ. de Botanique 17:217, 1903. **EPITYPE designated here,** MBT385828, to support the lectotype**:** strain JEL 347h, preserved as metabolically inactive, cryopreserved and deposited in CZEUM (University of Michigan), this publication.

Description: Light tan to pink white in culture. Sporangium: hyaline, globose, subspherical, 17-22 µm (sometimes up to 35 µm); walls smooth. Apophysis: fusiform, subspherical, pyramidal and variable with rhizoids extending laterally and dichotomously branched. Rhizoids: originate laterally from apophysis but can extend also from basal portion, extensive, delicate, finely branched, and pointed at the tips. Discharge: from single inoperculate pore, apical, subapical, lateral or basal. Zoospores: elongate or ovoid 2-3 µm x 3-4 µm; contain a single large colorless lipid globule; Resting spores: 11-20 µm x 8-14 µm.

Substrate: Saprotrophic on insect exuviae, chitin, cellulose, pollen, keratin, and depleted filaments of *Aphanomyces*.

Designated Habitat of Type: Water, Denmark.

Comments: Peterson (1903) mentions FIGS. 1 and 2 but did not explicitly designate a type nor indicate whether or not the figures were based on a single gathering. Thus, FIG.1 from Petersen (1903) is here designated as the lectotype. *A culture is selected as an epitype in support of the lectotype as allowed (Turland et al., 2018).*

Sparrow (1937) reported resting spores formed sexually by rhizoidal anastomosis between contributing thalli (Sparrow 1937); but because observations were of a mixed culture, the origin of the resting spores cannot be definitively determined; germination was not observed. Although the original description (Petersen, 1903) did not describe basal zoospore discharge, Sparrow indicated basal discharge in this species (Sparrow, 1960). We did not observe basal discharge in strain JEL 347h. The empty sporangium does not collapse (Fig. 3l).

Rhizoclosmatium aurantiacum Sparrow, Proc. Amer. Phil. Soc. 78: 40, FIGS 14-17, 1937.
Mycobank MB250877
= *Rhizoclosmatium globosum* H. E. Petersen 1903, pro parte, Journ. de Botanique 17: 216.
Typification: DENMARK, Gribskov, from water on exuviae of caddisfly. **LECTOTYPE designated here,** MBT385829, Plate 2 FIG. 17 in F. K. Sparrow. Proc. Amer. Phil. Soc. 78: 40; Plate 2, not paginated, 1937.

Description: Sporangium: orange, globose, smooth surface, 27-38 µm in diam. Apophysis: broadly fusiform with rhizoids branching from lateral sides. Rhizoids: delicate, extensive, and branched. Discharge: from single inoperculate pore, basal near the apophysis. Zoospores: elliptical 2.5 x 2.0 µm; contain small orange lipid globule. Resting spores: not observed.

Substrate: Saprotrophic in water on insect exuviae, chitin, cellulose, and pollen.

Designated Habitat of Type: Denmark, water on insect exuviae.

Comments: Petersen (1903) included orange thalli among his circumscription of *R. globosum*, considering them variants due to age or other environmental factors. Sparrow (1937) recognized an orange-colored *Rhizoclosmatium* as a new species. When Sparrow (1937) described *Rhizoclosmatium aurantiacum,* he did not explicitly designate a type, but FIGS. 14-17 were indicated and mentioned. However, these illustrations were from material from two different gatherings (FIGS. 14 and 17 from Danish material and FIG. 15 from United States material). Thus from among these figures, a single illustration (FIG. 17) from a single gathering in Denmark is designated here as the lectotype of *Rhizoclosmatium aurantiacum*.

Although we have isolated orange-colored strains of *Rhizoclosmatium*, none have produced a basal discharge pore near the apophysis as Sparrow (1937) describes for *R. aurantiacum*.

Rhizoclosmatium marinum Kobayasi and M. Ookubo, Bull. Nat. Sci. Mus., Tokyo, N.S. 1, 2 (35): 68, FIG. 7, 1954.

Mycobank MB305143

Typification: JAPAN. CHIBA PREFECTURE: Anegasaki, from marine waters on *Codium fragile* **LECTOTYPE designated here,** MBT385830, FIG. 7 in Kobayasi and Ookubo. Bull. Nat. Sci. Mus., Tokyo, N.S. 1, 2 (35): 69, 1954.

Description: Sporangium: hyaline; subglobose, oval, ellipsoidal; 30-40 x 30-60 µm. Apophysis: relatively large, globose, fusiform, transversely elongated or irregular. Rhizoids: coarse, extensive, sometimes thick at the base, two to three axes branching from the base of the apophysis. Discharge: through inoperculate basal pore into vesicle where zoospores swarm before swimming away. Zoospores: ellipsoidal 7 x 5 µm. Resting spores: not observed.

Substrate: Parasitic on the marine green alga, *Codium fragile.*

Designated Habitat of Type: Anegasaki, along coastal region of Chiba Prefecture, Honshu, Japan.

Comments: Although the authors did not explicitly designate a type, they mention FIG. 7, which is designated here as the lectotype. Because of its parasitic nature and marine habitat, this is a questionable species of the genus. Isolation and culture of this species are needed to allow the molecular and morphological analyses required to determine its relationship.

Rhizoclosmatium hyalinum Karling, Sydowia 20:101, FIGS. 48-59, 1967.

Mycobank MB338322

Typification: NEW ZEALAND. Dunedin, from water in outdoor tub with *Elodea* at University of Otago. **LECTOTYPE designated here,** MBT385831**,** Plate XIX FIG. 55 in Karling. Sydowia 20: 101 Plate 19, not paginated, 1967.

Description: Sporangium: hyaline; ovoid and slightly flattened at base, 38-45 x 48-64 µm, sometimes lobose. Apophysis: irregular, elongated transversally, 8-10 x 17-23 µm, subspherical 12-18 µm; rhizoids arising from several points on periphery. Rhizoids: arise from several points on periphery of apophysis, coarse, sparingly branched, extending up to 250 µm; thick walled and appearing stiff. Discharge: basal or lateral, inoperculate; discharge pore large, up to 18 µm diam. or discharge tube 3-4 µm diam. 4-12 µm long; containing a gelatinous plug; zoospores cleaved in sporangium and released externally into a vesicle where they swarm before swimming away. Zoospores: spherical 4.6-5 µm diam.; single large lipid globule. Resting spores: smooth, thick hyaline wall, subspherical 26-30 µm diam.; ovoid 20-26 x 30-34 µm; germination not observed.

Substrate: Saprotrophic on insect exuviae and chitin.

Designated Habitat of Type: Outdoor tub with *Elodea*, Botany Department, University of Otago, Dunedin, New Zealand.

Comments: No type was explicitly designed by author (Karling, 1967) but FIGS. 48-59 were indicated and mentioned (Articles 40.3, Turland et al., 2018). Valid publication of species on or after 1 January 1958 requires indication of type (Article 40.1), which is done here with a lectotype selected as a single illustration from among FIGS. 48-59 as permitted (Articles 40.3, 40.4). In discussion of this species, Karling (1967) emphasized the large size of the apophysis in relationship to the sporangium and the conspicuous enlarging primary nucleus during development of the incipient sporangium.

Rhizoclosmatium sparsum M. J. Powell and Letcher, *sp. nov.*

Mycobank 829907 Fig. 4

Typification: UNITED STATES, Alabama, Tuscaloosa, The University of Alabama, smaller Marr's Spring pond. From an aquatic sample containing bladderwort collected by M. J. Powell and baited with chitin, strain MP 56 isolated by M. J. Powell, HOLOTYPE Fig. 4G, this publication.

Ex-Type Strain: MP 56 deposited in CZEUM (University of Michigan)

Etymology: Latin *sparsum*, spread out, referring to the rhizoids.

Description: Sporangium: hyaline; spherical, 25-40 µm diam. Apophysis: spherical, 6-8 µm diam.; broadly ellipsoidal 4-5 x 7-8µm; prolate spheroidal, 6-8 x 4-5 µm; rhizoids arising from several points on the periphery or basally. Rhizoids: thin, sparsely and widely branched, extending up to 100 µm; septum dome-shaped protruding into the sporangium. Discharge: apical or subapical inoperculate pore; zoospores cleaved in sporangium and slowly swim inside before being released externally into a vesicle, where they swarm before swimming away. Zoospores: spherical 4 µm diam., to broadly ellipsoidal 4 µm x 5 µm, contain a single lipid globule. Resting spores: not observed.

Substrate: Saprotrophic on chitin and pollen.

Designated habitat of type: Marr's Spring, The University of Alabama, Tuscaloosa, AL.

Comments: Differs from *R. globosum* by production of less dense and more widely and sparsely branched rhizoids (Fig. 4D-H) with more trunk-like-extensions from the apophysis, and more oblong to tuberous apophysis (Fig. 4G-I, K). Zoospores are spherical to broadly oval when swimming (Fig. 4A) in contrast to the more elongate zoospore of *R. globosum.*

Additional specimens examined: UNITED STATES, North Carolina, Rutherford County, Broad River at base of Bill Mountain. From aquatic sample containing plant roots covered with periphyton collected by W. H. Blackwell and baited with chitin, strain WB 266C isolated by M. J. Powell; UNITED STATES, Alabama, Tuscaloosa, Marr's Pond. From aquatic sample baited with pollen, strain MP 46 isolated by M. J. Powell.

GenBank sequences of ex-type strain MP 56: JX905524 (28S rDNA), JX905553 (ITS1-5.8S-ITS2).

Rhizoclosmatium umbonatum Letcher, Longcore, and M. J. Powell, sp. nov.

Mycobank 829908 Fig. 5

Typification: UNITED STATES, Alabama, Wheeler National Wildlife Refuge. From an aquatic sample collected 9 August 2009 by B. Swan and baited with pollen; strain MP 44 isolated by M. J. Powell, HOLOTYPE Fig. 5I, this publication.

Ex-Type Strain: MP 44 deposited in CZEUM (University of Michigan)

Etymology: Latin *umbonatum*, indicating a rounded protuberance on the developing sporangium, from germling to maturity, where the discharge pore occurs.

Description: Sporangium: light orange to tan, spherical, 45-50 µm diam. at maturity; germlings, developing sporangia, and mature sporangia have an umbo that occurs where the discharge pore will later develop. Apophysis: variable, spherical, elongate, angular, often lobed and compound; rhizoids arising from several points on the periphery. Rhizoids: coarse, densely branched, extending up to 75 µm. Discharge: apical to subapical inoperculate discharge pore containing a thick gelatinous plug; zoospores cleaved within sporangium, slowly swarm inside, and released externally into a vesicle where they swarm before swimming away. Zoospores: spherical 4 µm diam., containing a single lipid globule. Resting spores: Not observed.

Substrate: Chitin and pollen

Designated Habitat of Type: Waters edge at Wheeler National Wildlife Refuge, AL.

Comments: This new species differs from other described species of *Rhizoclosmatium* in the persistence of an unexpanded portion of the zoospore cyst throughout development of the zoosporangium. In early development, the encysted zoospore produces a germ tube (Fig. 5B). The proximal portion of the germ tube expands (Fig. 5C), becomes confluent with the basal part of the encysted zoospore, and forms the incipient sporangium (Fig. 5 D-F). The apical portion of the zoospore cyst does not expand (Figs. 5C-E), remaining on the sporangium as rounded protuberance, the umbo (Fig. 5F). The distal portion of the germ tube develops into the rhizoidal system (Figs. 5C-F) with the apophysis enlarging after the formation of the incipient sporangium (Fig. 5G). As the thallus continues developing, the umbo remains, although it diminishes in size as the sporangium expands and partially incorporates it (Fig. 5F-H, J). Because the umbo is located at only one site on the sporangium, presumably at the location of the discharge pore, it is more difficult to locate on mature sporangia (Fig. 5J) than at earlier stages (Fig. 5F, G).

Additional specimens examined: UNITED STATES, Alabama, Hale County, Oakmulgee District of Talladega National. From dragonfly wings collected in a vernal pool aquatic sample, strain WJD 185 isolated by W. J. Davis.

GenBank sequences of ex-type strain MP 44: KF257907 (28S rDNA).

Rhizoclosmatium umbonatum Letcher, Longcore, and M. J. Powell var. *sphaericum* Letcher, Longcore, and M. J. Powell, var. nov.

Mycobank 829909 Fig. 6

Typification: UNITED STATES, Maine, Hancock County, Mud Pond, aquatic sample baited with cellulose; strain JEL 128 isolated by J. E. Longcore, HOLOTYPE Fig. 6J, this publication.

Ex-Type Strain: JEL 128 deposited in CZEUM (University of Michigan)

Etymology: Latin *sphaericum*, to indicate the spherical shape of the apophysis, which is distinguishable from the irregular, often compound apophysis of *R. umbonatum*.

Description: Sporangium: dark orange, suboblate to spherical during development, spherical at maturity, 30-35 μm diam.; germlings, developing sporangia, and mature sporangia have an umbo that occurs where the discharge pore will develop. Apophysis: predominantly spherical, 8-10 μm diam., rarely multi-lobed; rhizoids arising from several points on the periphery including basally. Rhizoids: dense, thin, widely branched, extending up to 80 μm. Discharge: apical to subapical inoperculate discharge pore containing a thick gelatinous plug; zoospores cleaved in sporangium, slowly swarm inside, released externally into a vesicle where they swarm before swimming away. Zoospores: spherical 4 μm diam., to slightly oval, 5 μm long. Resting spores: not observed.

Substrate: Cellulose and chitin

Designated Habitat of Type: Acidic lake, Mud Pond, Hancock County, ME.

Comments: As in *R. umbonatum,* a vestige of the encysted zoospore persists on the sporangium (Fig. 6E, F). The predominantly spherical apophysis of *R. umbonatum* var. *sphaericum* (Fig. 6H, J) makes it distinct from *R. umbonatum* with a predominantly multi-lobed apophysis (Fig. 5I). Apophysis begins to form after the incipient sporangium has started to expand (Fig. 6D versus 6E).

Additional specimens examined: UNITED STATES, Maine, from aquatic sample collected in 2006 from Perch Pond, Old Town, Penobscot County and baited with chitin, strain JEL 516 isolated by J. E. Longcore. UNITED STATES, Maine, from aquatic sample collected 1 June 2013 from Perch Pond, Old Town, Penobscot County and baited with chitin, strain JEL 796 isolated by J. E. Longcore.

GenBank sequences ex-type strain JEL 128: MK328908 (28S rDNA).

Rhizoclosmatium persicum Letcher, Longcore, and M. J. Powell, sp. nov.

Mycobank 829910 Fig. 7

Typification: UNITED STATES, Alabama, Tuscaloosa County. From an aquatic sample collected at the Lake Nicol dam spillway and baited with cellulose; strain MP 067 isolated by M. J. Powell, HOLOTYPE Fig. 7C, this publication.

Ex-Type Strain: MP 067 deposited in CZEUM (University of Michigan)

Etymology: Latin *persicum*, referring to bright orange flesh of the fruit of the apricot tree, *Prunus armeniaca*.

Description: Sporangium: orange; spherical, typically 25-45 μm diam., sometimes 55 μm diam.; primary nucleus continues to enlarge and divides after sporangium expansion is complete. Germling: encysted zoospore produces a long germ tube with few lateral branches; tip of germ tube bifurcates branches; apophysis produced later. Apophysis: spherical, subspherical, oval, angular pyramidal, or campanulate; most rhizoids extend from the sides of the apophysis. Rhizoids: primary axes stout; rhizoids moderately dense, finely branched; dome-shaped septum delimits rhizoid system from sporangium. Discharge: subapical to lateral inoperculate discharge pore; zoospores cleaved in sporangium, swarm in sporangium prior to discharge, released externally into a vesicle where they continue to swarm before swimming away. Zoospores: ellipsoidal 4 μm diam. x 5 μm length, single lipid globule. Resting spores: not observed.

Substrate: Cellulose and chitin

Designated Habitat of Type: Dam spillway, Lake Nicol, Tuscaloosa County, AL.

Additional specimens examined: UNITED STATES, Alabama, Tuscaloosa County, Coker, Lake Lurleen. From bank surface water sample baited 27 February 2008 with cellulose, strain EL 102 isolated by E. Lefèvre. UNITED STATES, Alabama, Hale County, Oakmulgee District of Talladega National. From *Daphnia* collected in a vernal pool aquatic sample, strain WJD 187 isolated by W. J. Davis; UNITED STATES, North Carolina, Rutherford County, Lake Lure. From aquatic sample containing green algae collected by W. H. Blackwell and baited with chitin, strain MP 14 isolated by M. J. Powell; UNITED STATES, North Carolina, Rutherford County, Lake Lure. From aquatic sample containing periphyton on aquatic plant stem collected by W. H. Blackwell and baited with chitin, strain MP 15 isolated by M. J. Powell.

GenBank sequences ex-type strain MP 067: KC691343 (28S rDNA).

Comments: The primary nucleus continues to enlarge as the sporangium expands (Fig. 7B). In the later production of an apophysis on the germ tube (Fig. 7A versus Fig. 8B) and more globose to pyramidal-shaped apophysis (Fig. 7B, C, D, F versus Fig. 8C, D, H), *R. persicum* differs from *R. pessaminum*. Zoospore discharge from a lateral pore is shown in Fig. 7D-E.

Rhizoclosmatium pessaminum Letcher, Longcore, and M. J. Powell, sp. nov.

Mycobank 829911 Fig. 8

Typification: UNITED STATES, Maine, from aquatic sample collected from Perch Pond, Old Town, Penobscot County in 2014 and baited with chitin, strain JEL 823 isolated by J. E. Longcore, HOLOTYPE Fig. 8D, this publication.

Ex-Type Strain: JEL 823 deposited in CZEUM (University of Michigan)

Etymology: 'pessamin', from the Powhatan language, an Algonquin language of the eastern United States, referring to the orange fruit of the American persimmon, *Diospyros virginiana*.

Description: Sporangium: orange; spherical, 30-50 µm diam. Apophysis: prolate to transversally elongate, 8-10 x 18-20 µm; rhizoids arising from several points on the periphery. Rhizoids: moderately dense, finely branched, extending up to 120 µm. Discharge: subapical to lateral inoperculate discharge pore containing a thin gelatinous plug; zoospores cleaved in sporangium, swarm in sporangium prior to discharge, released externally into a vesicle where they continue to swarm before swimming away. Zoospores: broadly ellipsoidal 4 µm diam. x 5 µm length, containing one to two lipid globules. Resting spores: not observed.

Substrate: Chitin

Designated Habitat of Type: Edge of Lake, Fen, Perch Pond, ME.

Additional specimens examined: UNITED STATES, Maine, from aquatic sample collected from Perch Pond, Old Town, Penobscot County in 2015 and baited with chitin, strain JEL 849 isolated by J. E. Longcore.

GenBank sequences ex-type strain JEL 823: MK328911 (28S rDNA).

Comments: The common presence of two lipid globules in a zoospore (Fig. 8A) is unlike the other described species. The apophysis forms early in germling development (Fig. 8B), and varies in shape from transversely elongate (Fig. 8C, D), oval (Fig. 8G) to prolate (Fig. 8H). A domed-shaped septum delimits the rhizoids from the sporangium (Fig. 8G, H). Zoospore discharge is through a subapical to lateral pore (Fig. 8E-G). This chytrid produced sporangia of two distinct sizes, one being 30–35 µm diam., the other being 50–60 µm diam. (Fig. 8I). The smaller sporangia are usually the sporangia that complete zoosporogenesis (Fig. 8I). The larger sporangia most often abort before zoosporogenesis; partially collapsed large sporangia often retain granular material, although a very few large sporangia had cleaved contents. It is known that *R. globosum* becomes hypertrophied when infected with the endoparasite *Rozella rhizoclosmatii* (Letcher et al., 2017), but we observed no indication of infection in these large thalli.

KEY TO SPECIES OF *RHIZOCLOSMATIUM*

1. Parasitic on marine green alga . . . . *R. marinum*
1. Saprotrophic on chitin, pollen, cellulose or keratin 2
 2. Hyaline, rose-white to light tan in culture 3

 2. Light orange to dark orange in culture 5
  3. Sporangia ovoid and sometimes lobed; rhizoids thick-walled and coarse . . . . *R. hyalinum*
  3. Sporangia spherical to subspherical; rhizoids thin-walled and delicate 4
   4. Apophysis transversely elongate . . . . *R. globosum*
   4. Apophysis spherical, ovoid to longitudinally tuber-shaped . . . . *R. sparsum*
  5. Germling with a prominent apical umbo 6
  5. Germling lacking a prominent apical umbo 7
   6. Apophysis predominantly multilobed at maturity . . . . *R. umbonatum*
   6. Apophysis predominantly spherical to ovate at maturity . . . . *R. umbonatum* var. *sphaericum*
    7. Zoospore discharge pore typically basal . . . . *R. aurantiacum*
    7. Zoospore discharge pore typically other than basal 8
     8. Apophysis subspherical, oval to pyramidal with stout rhizoidal axes . . . . *R. persicum*
     8. Apophysis transversely elongate to prolate, rhizoids dense . . . . *R. pessaminum*

DISCUSSION

Of the now eight species in *Rhizoclosmatium*, we were able to compare the morphology of five in pure culture and provide 28S rDNA sequence information to aid in their identification. Our study of strains grown in pure culture confirms the common characteristics of *Rhizoclosmatium.* In all species the primary nucleus enlarged in the incipient sporangium and divided only after the sporangium reached full size, as reported in several other chytrid species (Antikajian, 1949). A rhizoidal apophysis and domed-shaped septum separating the sporangium and apophysis were produced. As typical for members of the Chytridiales (Letcher and Powell, 2014), rhizoids were finely branched and tapered toward the tips. Early reports of *Rhizoclosmatium* were based on growth on natural substrates, and Sparrow (1960) indicated that basal discharge was common (Sparrow, 1960). With the strains we observed in culture, the single inoperculate discharge pore varied in position from apical, subapical, to lateral, but we did not observe basal discharge. Consistent with prior observations, zoospore discharge was vesicular.

The major morphological differences between species delimited in this study are complexity of rhizoidal system, shape of apophysis, and sporangial size and color in culture. Because of the plasticity of thallus morphology that chytrids typically exhibit (Powell and Koch, 1977), variability in these features is common. Variation in apophysis and rhizoidal structure ascribed to some strains of *R. globosum* in earlier studies, however, may reflect the possibility that other researchers have also observed strains that are new species of *Rhizoclosmatium.* For example, Johnson (1973) noted that strains from Iceland he considered as *R. globosum* often had apophyses that were large, irregular and sometimes cylindrical. We have also discovered that a chytrid strain described as *Phlyctochytrium powhatanensis* ATCC 22918 (Roane, 1973) is actually a strain of *R. globosum* (Fig. 1), most likely earlier confused because *Phlyctochytrium* produces an inoperculate discharge pore and apophysate thallus. Interestingly the strain was isolated from a chitin containing organism, a rotifer (Roane, 1973), a substrate on which *Rhizoclosmatium* can be expected. Consistent with the molecular results, Barr (1980, pg. 2388) earlier reported this strain had a Group I zoospore.

Morphological and molecular phylogenetic analyses support the circumscription of four new species and one new variety in this genus. *Rhizoclosmatium sparsum* produces hyaline to light tan thalli, as in *R. globosum. Rhizoclosmatium umbonatum, R. persicum,* and *R. pessaminum* produce light orange to bright

orange thalli, as in *R. aurantiacum.* When Petersen (1903) originally described *Rhizoclosmatium globosum*, he included both white/rose-colored and bright-orange-colored forms. Both forms were commonly collected from the same site and could occur together on the same piece of chitin. Sparrow (1937) recognized that strains with orange thalli and orange lipid globules in zoospores were distinct from *R. globosum*, and described *R. aurantiacum* as a new species. Unlike our orange-colored strains, zoospore discharge in *R. aurantiacum* was described as basal, with the pore near the apophysis (Sparrow, 1937).

Barr and Hartmann (1976) first described the zoospore ultrastructure of *Rhizoclosmatium globosum* (strain BR 34–ATCC 22197), and Barr (1980) designated it as a group I zoospore among the *Chytridiales* types of zoospores. More recent ultrastructural and molecular phylogenetic studies have used differences in zoospore ultrastructure to delineate the family Chytriomycetaceae from the family Chytridiaceae in the Chytridiales (Letcher and Powell, 2014; Vélez et al., 2011). The *Rhizoclosmatium* strains we studied had zoospores with the features of a group I zoospore as Barr (1980) described and Letcher and Powell (2014) further defined. The only difference among strains was the position of the nucleus within the ribosomal aggregation. Among the orange pigmented strains, the nucleus was partially inserted into the ribosomal aggregation in *R. persicum* (strains MP 67, WJD 187) and *R. umbonatum* (strains MP 44, WJD 185); but among the more hyaline strains, such as *R. globosum* (strain WJD 111), the nucleus, although still partially inserted into the ribosomal aggregation, was not as deeply inserted. These results are consistent with Letcher and Powell's (2014) hypothesis based on analysis of character state evolution. They proposed that nuclear location in the zoospore is a neutral character in natural selection. In the last common ancestor of Chytridiales, the nucleus was located within the ribosomal aggregation; nuclear progressive movement toward the surface of the ribosomal aggregation and then to the periphery occurred in independent linages.

Chytrids are recognized as important degraders of refractory material including cellulose, chitin, keratin and pollen (Goldstein, 1960; Tribe, 1957). Recent ecological studies demonstrate that chytrids have a role in the 'Mycoloop" in transforming materials such as pollen, which many zooplankton cannot use, into a form which they can use, nutritionally rich chytrid zoospores (Kagami et al., 2007b, 2014, 2017). Species in the genus *Rhizoclosmatium* are considered chitin degraders (Sparrow, 1960), but based on our sampled strains, are also able to break down other substrates including pollen as well as keratin and cellulose-containing substrates. Descriptions of sites for collection of *Rhizoclosmatium* species often indicate an association with aquatic vegetation. Thus, *Rhizoclosmatium* species, which are common in aquatic habitats, may have a significant role in nutrient cycling and productivity of aquatic habitats. The complete genome of *R. globosum* (JEL 800) is available from the US DOE Joint Genome Institute, making it possible to determine its enzymatic capabilities (Mondo et al., 2017). With the widespread occurrence of *R. globosum*, its ease of culture, its generation time of ~20 hrs. at 23° C, and the availability of its genetic information, *R. globosum* can be a valuable model organism. The importance of the genus *Rhizoclosmatium* in nutrient cycling and sustainability in aquatic habitats is an important avenue to investigate further.

CONCLUSIONS

This study has shown that species diversity within *Rhizoclosmatium* is greater than earlier realized. Because chytrids exhibit thallus plasticity, identification of species based on morphology alone is problematic. Consequently, molecular phylogenetics inform our understanding of species distinctions within this genus. Significantly, zoospore ultrastructure is conserved within the genus. Surveys of chytrid biodiversity have demonstrated that a few chytrid species are essentially ubiquitous and many are scarce to rare (Davis et al., 2013; Letcher and Powell, 2001; Letcher et al., 2004, 2006, 2008a, 2008b). We have previously shown that for chytrid taxa that are common, local sampling tends to reflect global diversity (Davis et al., 2013; Letcher et al., 2008b). The geographic sampling in this study was somewhat limited,

predominantly southeastern and northeastern United States, yet species diversity was revealed. From this sampling we found that *R. globosum* was a common taxon and the newly described species were rarer. These results suggest that additional sampling could reveal greater diversity in this genus.

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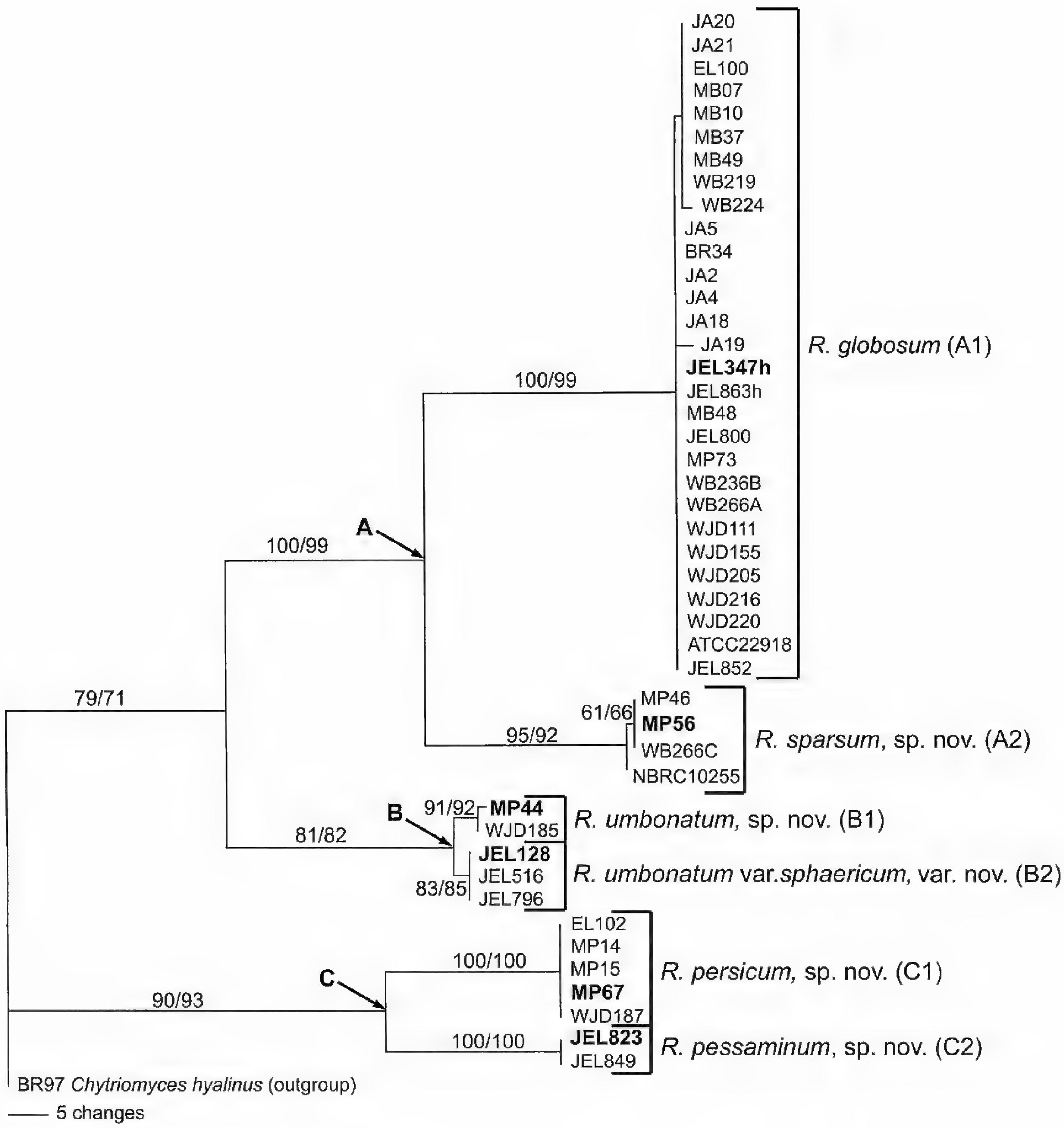

Figure 1. Molecular phylogenetic assessment. Phylogram inferred from strict consensus, maximum parsimony analysis of 45 strains of *Rhizoclosmatium* using 28S rDNA. Numbers at nodes are bootstrap support values (maximum likelihood/maximum parsimony). Three lineages are resolved (A, B, C), each with two sub-clades (A1, A2, B1, B2, C1, C2).

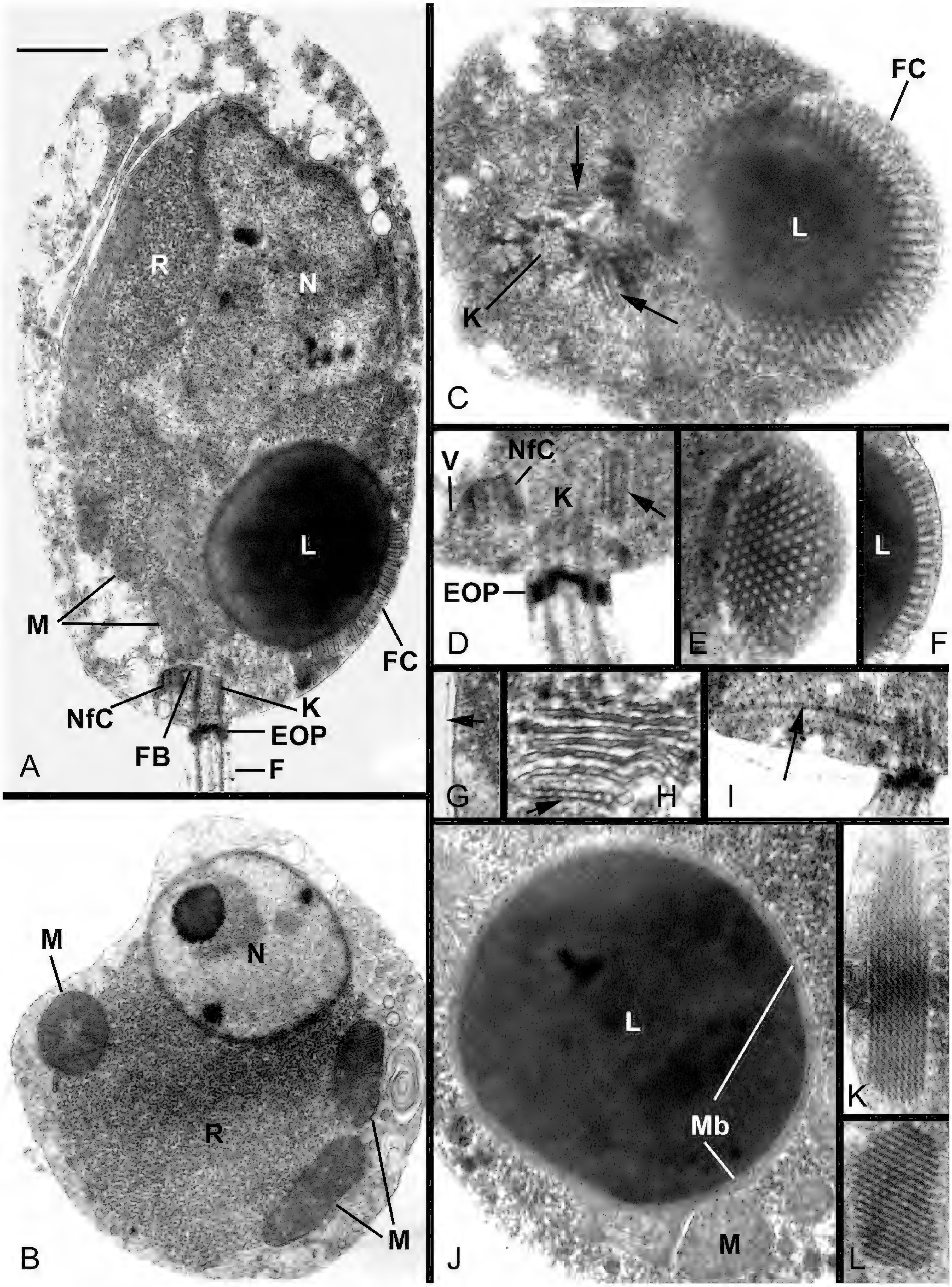

Figure 2. See caption on following page.

Figure 2. Ultrastructure of the *Rhizoclosmatium* species zoospore. A, C, E–H. *R. persicum* strain MP 67; B, K, L. *R. umbonatum* strain WJD 185; D, I. *R. globosum* strain WJD 111; J. *R. globosum* strain ATCC 22918. (A) Longitudinal section with aggregated ribosomes, a nucleus partially inserted into the ribosomal aggregation, a fenestrated MLC cisterna appressed to a single lipid globule, multiple mitochondrial profiles outside the ribosomal aggregation, a non-flagellated centriole adjacent to the kinetosome, the two connected by a fibrillar bridge, and an electron-opaque plug in the base of the flagellum. (B) TS with the nucleus partially inserted in the ribosomal aggregation, and multiple mitochondrial profiles outside the aggregation. (C) TS through the kinetosome, illustrating the KAS as a set of plates (arrows), and an oblique section through the fenestrated MLC cisterna. (D) LS illustrating a veil adjacent to the non-flagellated centriole and the KAS adjacent to the kinetosome (arrow). (E) Face view and (F) LS through fenestrated MLC cisterna. (G) Cell coat (arrow) adjacent to plasma membrane. (H) Golgi apparatus; notice partitioned cisterna (arrow). (I) LS illustrating microtubular root (arrow) extending from kinetosome toward fenestrated MLC cisterna. (J) Microbody appressed to the lipid globule. (K) LS through paracrystalline inclusion. (L) TS through paracrystalline inclusion. Scale bar = 1.0 µm (A, B, C, E, F), 0.5 µm (I), 0.4 µm (J, K, L), 0.3 µm (H), 0.25 µm (D, G). Abbreviations: EOP, electron-opaque flagellar plug; F, flagellum; FB, fibrillar bridge; FC, fenestrated MLC cisterna; K, kinetosome; KAS, kinetosome-associated structure; L, lipid globule; LS, longitudinal section; M, mitochondrion; MB, microbody; MLC, microbody-lipid globule complex; N, nucleus; NfC, non-flagellated centriole; R, ribosomal aggregation; TS transverse section; V, veil.

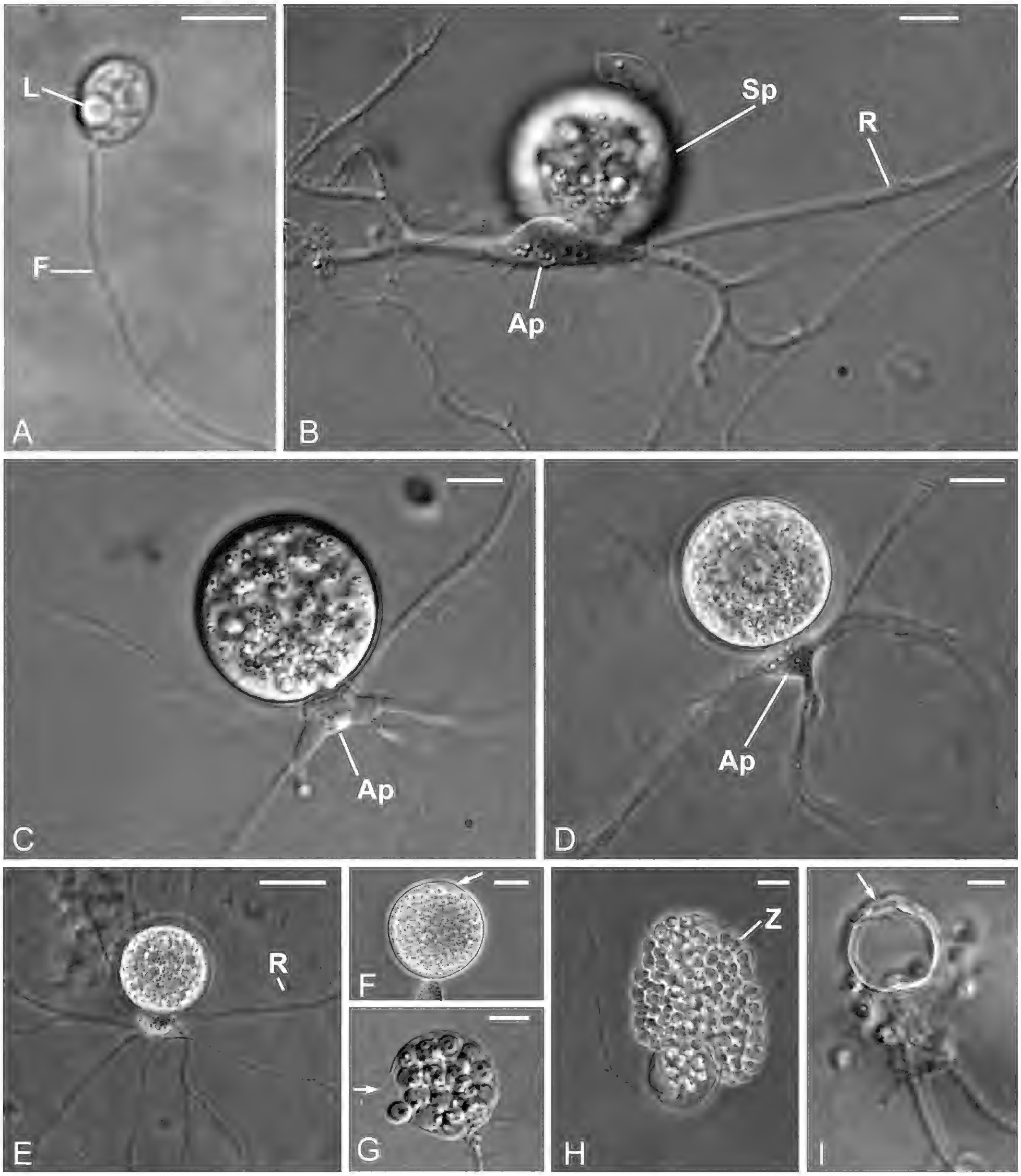

Figure 3. Thallus morphology of *R. globosum*, strain JEL 347h. (A) Motile zoospore with a single lipid globule and posterior flagellum. (B) Developing sporangium with subsporangial apophysis and rhizoids originating from multiple points on the apophysis. (C–E) Developing sporangia, the subsporangial apophyses having variable morphology. (F) Maturing sporangium with a hyaline plug located at the apical discharge pore (arrow). (G) Mature sporangium with a lateral discharge pore (arrow). (H) Vesicular discharge of zoospores. (I) Empty sporangium with apical discharge pore (arrow). Scale bar = 20 µm (E), 10 µm (B, C, D, F, G, H, I), 5 µm (A). Abbreviations: Ap, apophysis; F, flagellum; L, lipid globule; R, rhizoid; Sp, sporangium; Z, zoospore.

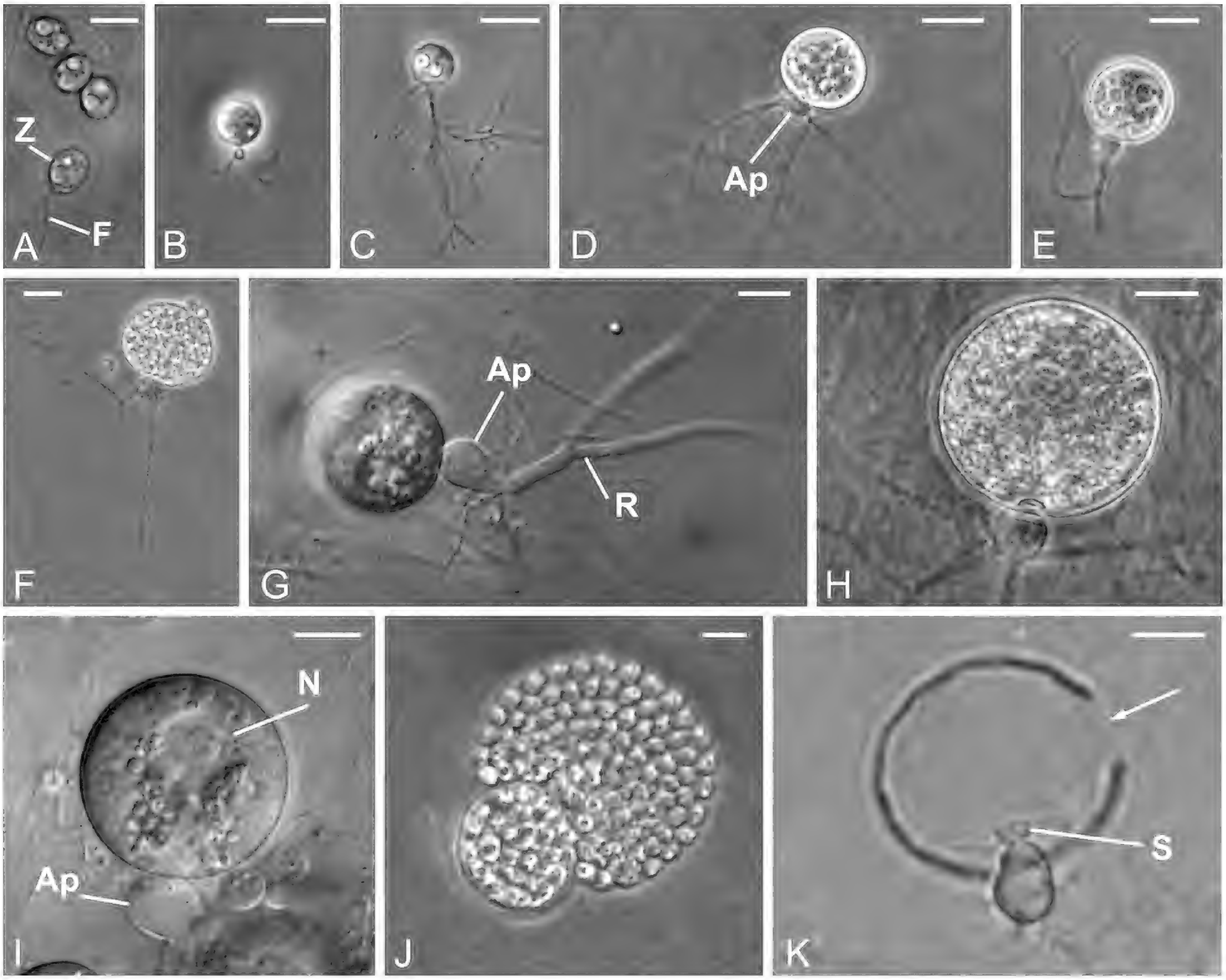

Figure. 4. Thallus morphology of *R. sparsum* sp. nov., strain MP 56. (A) Broadly oval zoospores. (B) Young sporangium with rhizoidal system extending from small, round apophysis. (C) Germling with long rhizoid. (D) Developing thallus with elongate apophysis. (E) Developing thallus with tuberous apophysis and basal, trunk-like, sparely branched rhizoid system. (F) Mature thallus with apical discharge pore; sporangium with cleaved zoospores; rhizoidal system finely branched with widely spaced rhizoids arising from base of spherical apophysis. (G) Developing thallus with tuberous apophysis and basal, trunk-like rhizoidal axis. (H) Mature, spherical sporangium; widely spaced rhizoidal axes emanating from total surface of subspherical apophysis. (I) Primary nucleus in sporangium is enlarged; apophysis subspherical. (J) Vesicular discharge of zoospores. (K) Empty sporangium with lateral discharge pore (arrow); dome-shaped septum extending from tuberous apophysis. Scale bar =10 µm (B-K), 5 µm (A). Abbreviations: Ap, apophysis; F, flagellum; N, large primary nucleus; R, rhizoid; S, septum; Z, zoospore.

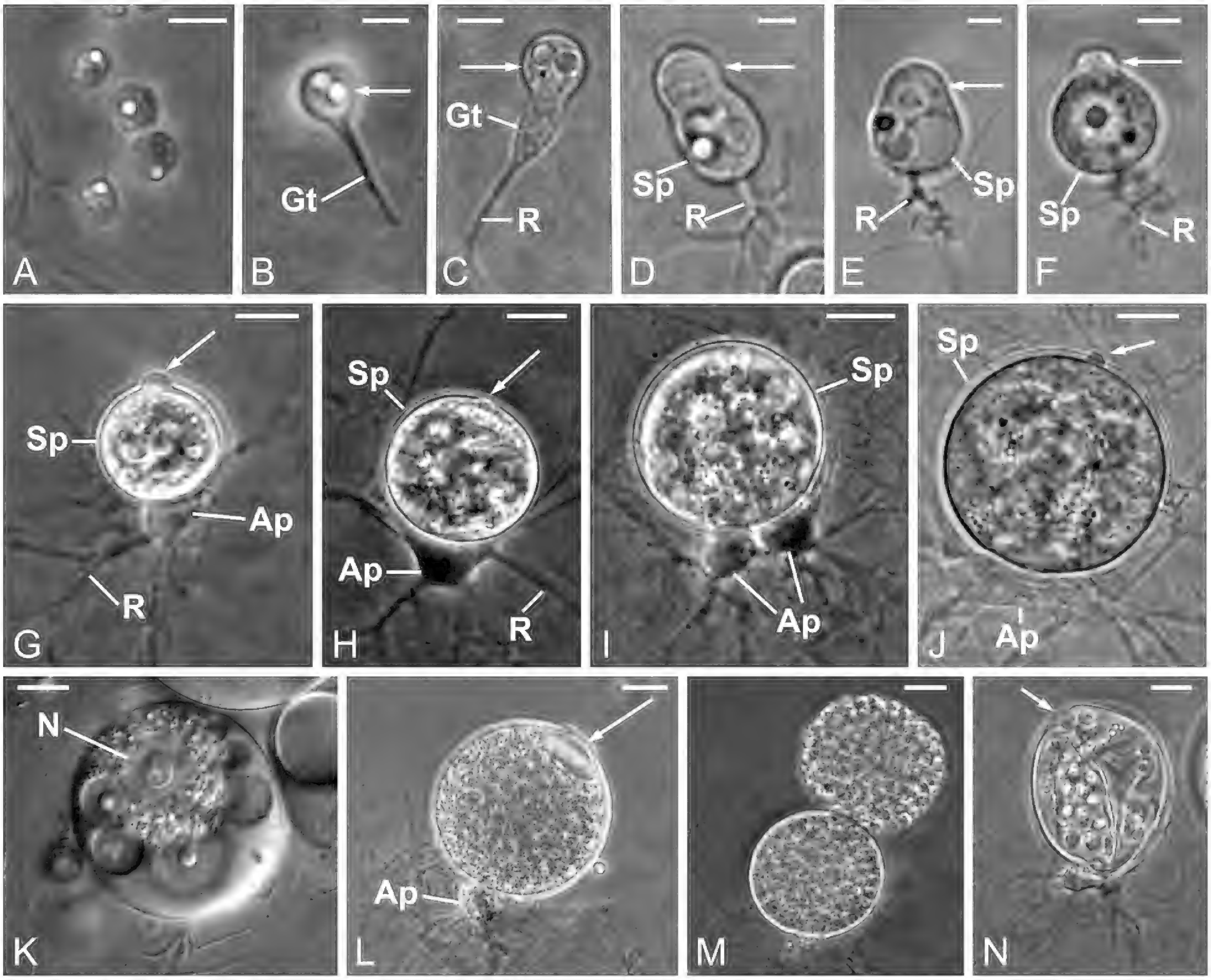

Figure 5. Thallus morphology of *R. umbonatum* sp. nov., strain MP 44. (A) Zoospores with single lipid globule. (B) Germling; encysted zoospore (arrow) with germ tube. (C) Germling; proximal portion of germ tube swells and becomes confluent with a portion of the encysted zoospore (arrow); distal portion of germ tube becomes rhizoid. (D, E, F) Early thallus development; apical portion of encysted zoospore not totally incorporated into sporangium and remains as an umbo (arrow); sporangium and rhizoid develop; apophysis not enlarged yet. (G) Immature thallus with spherical sporangium bearing an apical umbo (arrow); apophysis enlarged. (H) Developing thallus; rhizoids originate from multiple points on the apophysis; remnant of umbo (arrow). (I) Developing sporangium with multi-lobed apophysis. (J) Maturing sporangium with apophysis and remnant of umbo (arrow). (K) Maturing sporangium with large primary nucleus. (L) Mature sporangium with thick hyaline plug in the apical discharge pore (arrow); one lobe of apophysis visible. (M) Vesicular zoospore discharge. (N) Almost empty sporangium; subapical discharge pore (arrow). Scale bar = 5 µm (A–F), 10 µm (G–N). Abbreviations: Ap, apophysis; Gt, germ tube; N, large primary nucleus; R, rhizoid; Sp, sporangium.

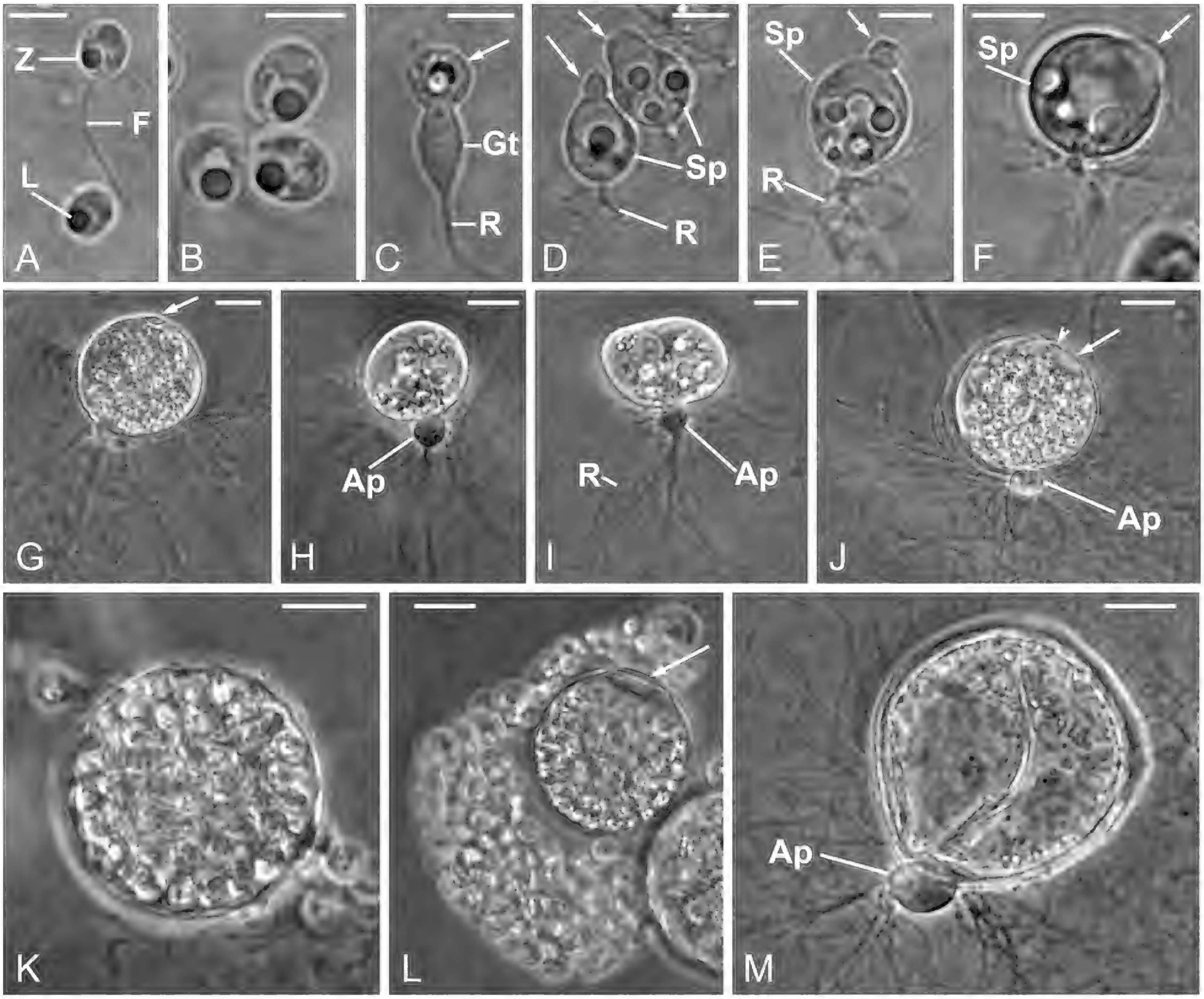

Figure 6. Thallus morphology of *R. umbonatum* var. *sphaericum*, var. nov., strain JEL 128. (A) Motile zoospores each with a single lipid globule and posterior flagellum. (B) Encysting zoospores each containing a single lipid globule. (C) Germling; encysted zoospore (arrow) with germ tube; proximal portion of germ tube expands and the distal portion develops the rhizoidal axis. (D) Two developing thalli; encysted zoospore (arrow) partially confluent with expanded portion of germ tube becoming the sporangium; apical portion of encysted zoospore only partially expanded, remaining as an apical umbo (arrow); tip of germ tube developing rhizoids. (E) Immature sporangium with an apical umbo (arrow). (F) Developing sporangium with a subapical umbo (arrow). (G) Maturing sporangium with remnant of apical umbo (arrow). (H) Maturing, subglobose sporangium with spherical apophysis. (I) Maturing, suboblate sporangium with spherical apophysis. (J) Maturing, spherical sporangium with spherical apophysis and subapical hyaline plug (arrow) at site of discharge pore; remnant of apical umbo is visible (arrowhead). (K) Mature sporangium with cleaved zoospores. (L) Vesicular discharge of zoospores; arrow indicates discharge pore. (M) Empty sporangium with spherical apophysis. Scale bar = 5 µm (A–E), 10 µm (F–M). Abbreviations: Ap, apophysis; F, flagellum; Gt, germ tube; L, lipid globule; R, rhizoid; Sp, sporangium; Z, zoospore.

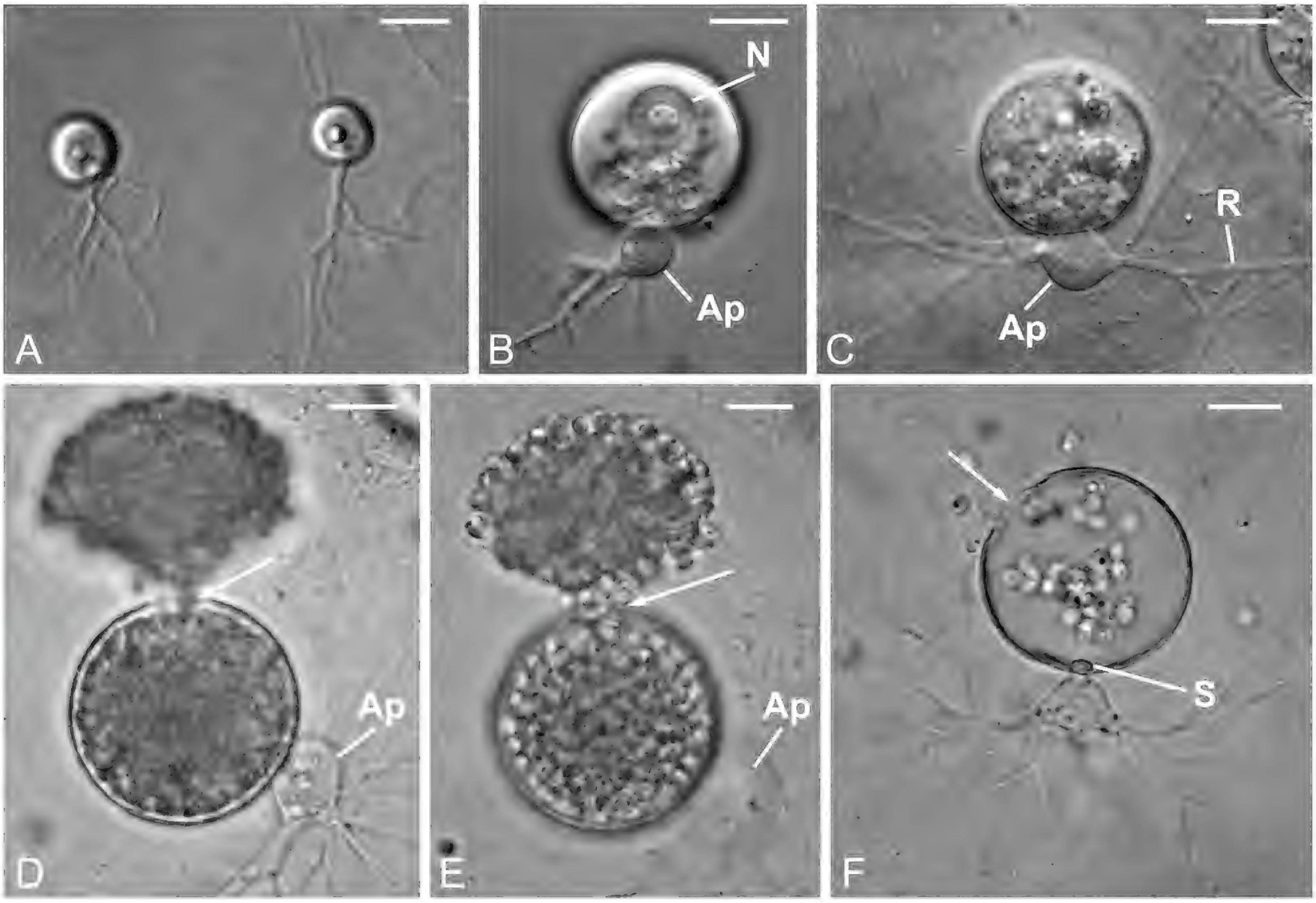

Figure 7. Thallus morphology of *R. persicum* sp. nov., strain MP 67. (A) Two germlings early rhizoidal development. (B) Developing thallus with large primary nucleus in sporangium; subglobose apophysis. (C) Maturing thallus; spherical sporangium with ovate apophysis; stout rhizoidal axes emanating from sides of apophysis. (D-E) Two focal levels through discharging sporangium showing location of angular pyramidal apophysis and lateral position of discharge pore (arrow); zoospores released from sporangium as a mass before swimming away. (F) Thallus after zoospore discharge revealing subapical discharge pore (arrow), dome-shaped septum between the sporangium and apophysis, and fine rhizoids extending from the sides of the campanulate-shaped apophysis. Scale bar = 10 µm (B-F), 5 µm (A). Abbreviations: Ap, apophysis; N, large primary nucleus; R, rhizoid; S, septum; Z, zoospore.

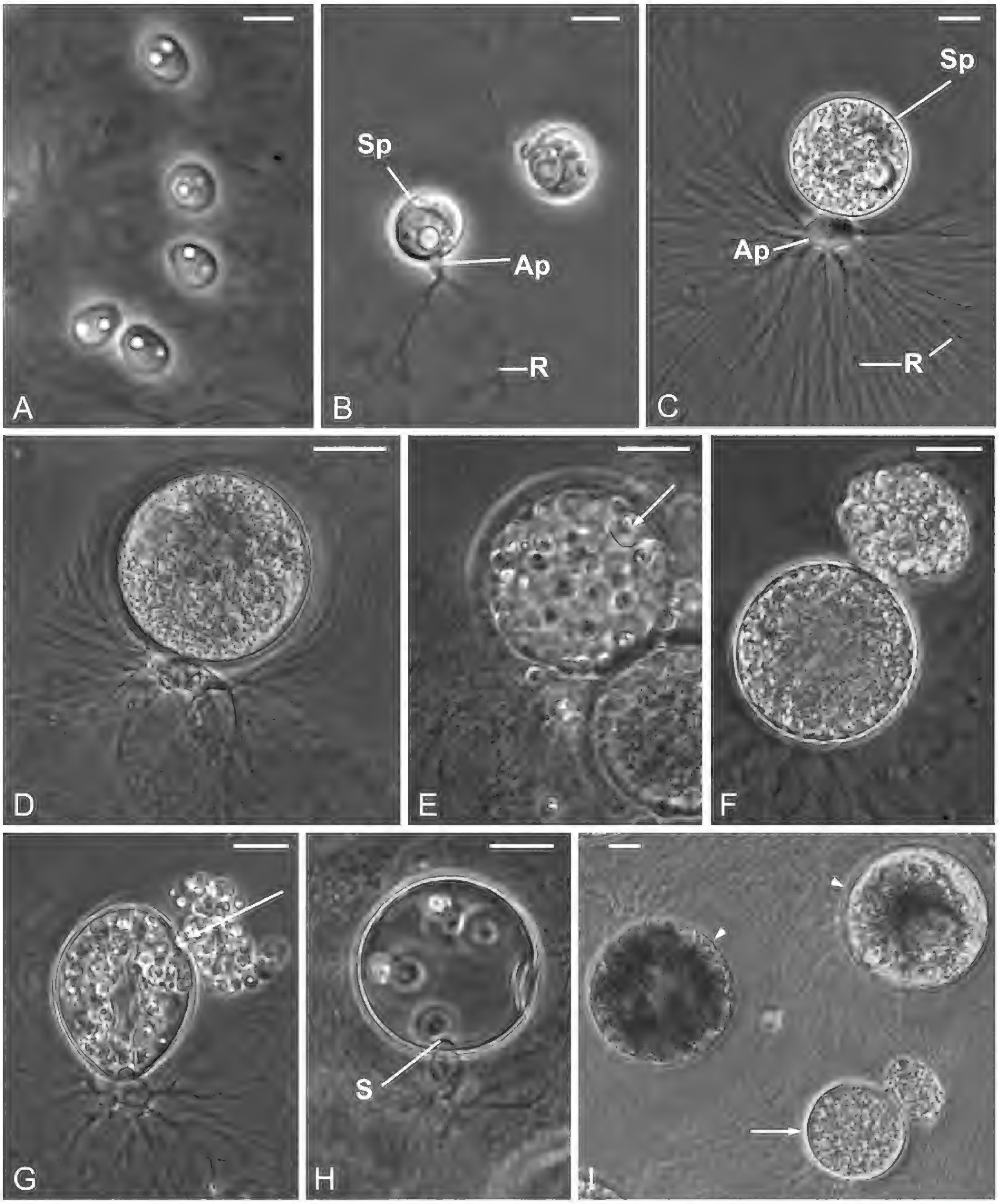

Figure 8. Thallus morphology of *R. pessaminum* sp. nov., strain JEL 823. (A) Motile zoospores containing two lipid globules. (B) Germling with spherical sporangium, a subsporangial apophysis, and rhizoids. (C) Immature sporangium with transversely elongate apophysis. (D) Maturing sporangium. (E) Mature sporangium with cleaved zoospores; arrow indicates location of discharge pore. (F) Vesicular discharge of zoospores. (G) Late in zoospore discharge; arrow indicates location of discharge pore. (H) Empty sporangium, dome-shaped septum, prolate apophysis. (I) Two sporangial sizes indicated; smaller sporangium (arrow) is beginning vesicular discharge of zoospores; larger sporangia (arrowheads) without cleaved contents. Scale bar = 5 µm (A, B), 10 µm (C–I). Abbreviations: Ap, apophysis; R, rhizoid; S, septum; Sp, sporangium.

Table 1. Strains used in phylogenetic analysis			
Strain	Location of Collection	Substrate	GenBank
			28S Accession #
	Rhizoclosmatium globosum		
JA 20	Vernal Pool, TNF, Hale County, AL[1]	Cellulose	KU721082
JA21	Vernal Pool, TNF, Hale County, AL[1]	Keratin	KU721083
EL100	Lake Lurleen, Coker, AL	Cellulose	JN049527
MB07	Lake Lurleen #2, Coker, AL	Pollen	KC691322
MB10	Lake Lurleen #2, Coker, AL	Chitin	DQ273969
MB37	Lake Lurleen #4, Coker, AL	Chitin	KC691329
MB49	Lake Lurleen #5, Coker, AL	Chitin	*MK328900
WB219	Lake Lurleen, Coker, AL	Chitin	KC691360
WB224	Rainey Pond, Cottondale, AL	Chitin	KC691361
JA5	Lake Nicol, Tuscaloosa, AL	Chitin	KC691316
BR34=	Lake, Gatineau Park, Quebec CANADA	Pollen	JN941008
ATCC2219			
JA2	Lake Nicol, Tuscaloosa, AL	Cellulose	KC691313
JA4	Lake Nicol, Tuscaloosa, AL	Chitin	KC691315
JA18	Vernal Pool,TNF, Hale County, AL[1]	Cellulose	KU721080
JA19	Vernal Pool,TNF, Hale County, AL[1]	Pollen	KU721081
JEL347h	Fen, Perch Pond, Penobscot Co, ME	chitin	DQ273769
JEL863h	Fen, Perch Pond, Penobscot Co, ME	Chitin	KX354826
MB48	Lake Lurleen #5, Coker, AL	Cellulose	KC691131
JEL 800	Fen, Perch Pond, Penobscot Co, ME	Chitin	*MK543211
MP73	Creek culvert, Wagarville, AL	Cellulose	*MK328902
WB236B	Lake Nicol, Tuscaloosa, AL	Chitin	KC691365
WB266A	Broad River, near Bills Mt., Lake Lure, NC	Chitin	*MK328903
WJD111	Lake Lurleen, Coker, AL	Keratin	KC691374
WJD155	Singer Lake Bog, Summit County, OH	Cellulose	*MK328904
WJD205	Vernal Pool, TNF, Hale County, AL[1]	Chitin	KU721085
WJD216	Vernal Pool, Godfrey Drive, Orno, ME	Cellulose	*MK328905
WJD220	Fen, Perch Pond, Old Town, ME	Cellulose	*MK328906
ATCC22918	Camp Powhatan, Pulaski County, VA	Rotifer	*MK328907
JEL 852	Fen, Perch Pond, Penobscot Co, ME	Chitin	*MK328901
	Rhizoclosmatium sparsum, sp. nov		
MP46	Marr's Spring, Tuscaloosa, AL	Pollen	JX905523

MP56	Marr's Spring, Tuscaloosa, AL	Chitin	JX905524
WB266C	Broad River, near Bills Mt., Lake Lure, NC	Chitin	JX905528
NBRC10255	Sugadaira Montane, Research Center, Japan		NBRC10255
	Rhizoclosmatium umbonatum, sp. nov.		
MP44	Wheeler Wildlife Refuge, AL	Pollen	KF257907
WJD185	Vernal Pool,TNF, Hale County, AL[1]	Dragonfly wing	KU721087
	Rhizoclosmatium umbonatum		
	var. *sphaericum,* var. nov.		
JEL128	Lake, Mud Pond, Hancock Co., ME	Cellulose	*MK328908
JEL516	Fen, Perch Pond, Penobscot Co., ME	Chitin	*MK328909
JEL796	Fen, Perch Pond, Penobscot Co., ME	Chitin	*MK328910
	Rhizoclosmatium persicum, sp. nov.		
EL102	Lake Lurleen, Coker, AL	Cellulose	JN049529
MP14	Lake at Lake Lure, NC	Chitin	*MK328914
MP15	Lake at Lake Lure, NC	Chitin	*MK328915
MP67	Lake Nicol, Tuscaloosa, AL	Cellulose	KC691343
WJD187	Vernal Pool,TNF, Hale County, AL[1]	Daphnia	KU721088
	Rhizoclosmatium pessaminum, sp. nov.		
JEL823	Fen, Perch Pond, Penobscot Co., ME	Chitin	*MK328911
JEL849	Fen, Perch Pond, Penobscot Co., ME	Chitin	*MK328913
	Outgroup		
BR97 =	*Chytriomyces hyalinus*:	Moribund	AY439074
ATCC 24931	Ramsayville Marsh, near Ottawa CANADA)	green alga	
	1 Oakmulgee District of the Talladege National Forest (TNF)		
	*Indicates newly generated sequences		

The origin of *Juniperus* x*pfitzeriana,* an allo-tetraploid hybrid of *J. chinensis* x *J. sabina*

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ABSTRACT

Juniperus x*pfitzeriana* is one of the most commonly cultivated junipers in the world. The origin of *J.* x*pfitzeriana* has remained speculative, but it is thought to be a hybrid of *J. chinensis* x *J. sabina.* nrDNA (ITS) and 4 chloroplast gene regions were sequenced from 14 *J.* x*pfitzeriana* cultivars from Windsor Gardens, UK, and compared with all *Juniperus*, sect. *sabina*, smooth leaf margin species. All of the 14 cultivars were identical in their chloroplast DNA and their cp DNA was identical to that of *J. sabina* var. *balkanensis*. In addition, 13 *J.* x*pfitzeriana* cultivars were allo-tetraploids with heterozygous bases at 5 to7 sites that distinguish *J. chinensis* and *J. sabina* var. *balkanensis*. These cultivars had identical nrDNA. Two of the 14 cultivars, 'Old Gold' and 'Sea Green', showed a slightly different nrDNA pattern, being homozygous at sites 410 and 1139, as found in *J. s.* var. *balkanensis*. The origin of *J.* x*pfitzeriana* is from a cross of a male, tetraploid *J. sabina* var. *balkanensis* and a female, tetraploid, *J. chinensis*, resulting in an allo-tetraploid, dioecious, *J.* x*pfitzeriana* (Spath) Schmidt. Published on-line www.phytologia.org *Phytologia 101(2): 164-174 (June 21, 2019).* ISSN 030319430.

KEY WORDS: *Juniperus* x*pfitzeriana*, x*media*, *J. chinensis*, *J. sabina* var. *balkanensis*, tetraploid, origin.

Juniperus x*pfitzeriana* is one of the most commonly cultivated junipers in the world (Krussmann, 1991, listed 28 cultivars). The origin of the group of cultivars treated as 'Pfitzers' is thought to have been from a cross of *J. chinensis* x *J. sabina* (Le Duc, et al. 1999; Krussmann 1991; van Melle 1947). Van Melle (1947) proposed the name *Juniperus xmedia* for *J. chinensis 'Pfitzeriana'*, having concluded that it was a hybrid. The missionary Armand David collected the seed from the Ho Lan (Helan) Shan, Inner Mongolia and sent seed back to France in the 1860s (van Melle 1947). Van Melle (1947) notes that, by the 1870s, the plants, obtained by growing the seeds, were cultivated 'extensively' in France and Belgium by nurserymen. The Spath nursery selected a male plant and named the cultivar 'Pfitzeriana' after W. Pfitzer, a nurseryman at Stuttgart (Den Oden and Boom, 1965). It is interesting that van Melle (1947) recognized several male and female cultivars as varieties, thus pfitzers are dioecious as are the putative parents, *J. chinensis* and *J. sabina* (Adams 2018). Van Melle (1947) recognized two male varieties: *J. xmedia* var. *pfitzeriana*, and var. *globosa*; and two female: var. *arbuscula* and var. *plumosa*. The seedlings grown in France and Belgium produced several 'sports' or somatic mutations, and these were further propagated by cloning to conserve the somatic mutation, to obtain commercially valuable cultivars. These seedlings matured to become male or female reproducing plants. This provides evidence that the natural hybrids in Ho Lan Shan, produced viable seed, yielding seedlings that later displayed the 'Pfitzer' hybrid phenotype.

Juniperus x*pfitzeriana* (Spath) Schmidt (Schmidt 1983) is widely accepted as the name of the 'Pfitzers', but *Juniperus xmedia* Van Melle is still in use, although the name has been rendered illegitimate because of prior usage of *J. media* V. D. Dmitriev (Le Duc et al. 1999, Czerepanov 1973). Lewis (1995) attempted to get the name *J. xmedia* conserved because of historical usage, but his proposal was rejected (Le Duc et al. 1999).

The volatile leaf oil of *J. chinensis* contains bornyl acetate and the oil of *J. sabina* includes sabinyl acetate. Fournier et al. (1991) reported that the volatile leaf oils of pfitzeriana cultivars contained both bornyl acetate and sabinyl acetate. Thus, the oils supported (but not proving) the origin from *J. chinensis* x *J. sabina*.

Le Duc et al. (1999) used RAPDs (Random Amplified Polymorphic DNAs) to ordinate *J. chinensis* (*Adams 6764-6766*, cv 'Kaizuka', cultivated at Northwest Normal University, Lanzhou, China), *J. sabina* var. *sabina* (*Adams 7611-7614*, Switzerland) and eight *J.* x*pfitzeriana* cultivars ('Fruitlandii', 'Gold Coast', 'Hetezii', 'Kallay's Compact', 'pfitzeriana Aurea', 'pfitzeriana Glauca' and 'Wilhelm Pfitzer', the cultivar of the type for *J.* x*pfitzeriana* by Schmidt, 1983. Using 122 RAPD bands, they found the x*pfitzeriana* samples ordinated intermediate between the putative parental species (*J. chinensis*, *J. sabina* var. *sabina*), as one would expect in hybrids (Adams1982). Again, these data supported (but did not prove) the origin from *J. chinensis* x *J. sabina*.

With the advances in DNA sequencing technology it is now possible to deduce the parents in conifer hybrids, and the inheritance of chloroplast (cp) in the Cupressaceae has been shown (Table 1) to be from the male, pollen parent (Adams 2019).

Scion Ltd., New Zealand recently made available materials from controlled crossings in *Hesperocyparis*, a genus closely related to *Juniperus*. Adams et al. (2016) analyzed 18 hybrids from a single, controlled cross, *H. arizonica* (male) x *H. macrocarpa* (female), and all 18 had perfect *H. arizonica* (paternal) chloroplast DNAs, confirming paternal inheritance of chloroplasts in *Hesperocyparis* (Table 2), and by inference, in the closely related genus, *Juniperus*.

Recently, it has been proved that genome size using flow cytometry (FC) was successfully used as a proxy for ploidy level in *Juniperus* (Farhat et al. 2019a, b). Therefore, the ploidy of Juniper hybrids can now be determined by FC. This is very important because it is known that several *J. chinensis* pfitzers are

tetraploid (Hall, et al. 1979). With the confluence of both DNA methodology and FC ploidy determination, this present us with a great opportunity to examine the origin of *J.* x*pfitzeriana*.

The purpose of the present research is to present new DNA sequencing utilizing both chloroplast and nuclear DNA in the determination of the origin of *J.* x*pfitzeriana*. We also present ploidy for *J.* x*pfitzeriana* cultivars and the putative parental species, *J. chinensis* and *J. sabina*.

Table 1. Inheritance of cp (chloroplasts) and mt (mitochondria) in conifers. ns = not studied. From Adams 2019, in part).

Cupressaceae	cp	mt	reference (see Adams 2019 for ref.)
Cunninghamioideae			
Cunninghamia konshii	mat	ns	Lu, et al. 2001
Sequoioideae			
Sequoia sempervirens	pat	pat	Neale, Marshall and Sederoff, 1989
Taxodioideae			
Cryptomeria japonica	pat, some mat leakage	ns	Ohba et al. 1971
Callitroideae			
Callitris (4 species)	pat	ns	Sakaguchi, et al. 2014
Cupressoideae			
Leyland cypress - *Callitropsis nootkatensis* x *Hesperocyparis macrocarpa*	4 plants: pat	pat	Kou, et al. 2014
	2 plants: mat	mat	
Calocedrus decurrens	pat	pat	Neale, Marshall and Harry, 1991
Chamaecyparis obtusa	pat, ~2.5% mat leakage	ns	Shirashi et al. 2001
Chamaecyparis obtusa x pisifera	pat	pat	Kondo, et al., 1998
Chamaecyparis lawsonia	pat	pat	Chesnoy, 1973
Platycladus orientalis	pat	pat	Chesnoy, 1969
Hesperocyparis arizonica x *Hesperocyparis macrocarpa*	pat	ns	Adams et al. 2018
Juniperus ashei, J. pinchotii, J. virginiana	pollen	pollen	Mohanty et al. 2016, ultrastructural presence of cp and mt in pollen was confirmed by TEM and DNA.

METHODS

Plant materials:

xpfitzeriana samples: Leaf samples were collected in Windsor Gardens, Windsor Great Park, Windsor, *SL4 2HT* UK from 14 *J.* x*pfitzeriana* cultivar accessions and immediately placed in activated silica gel for DNA sequencing and Flow Cytometry - ploidy determination (see Table 2).

Reference Species: *Juniperus chinensis*, *J. sabina* var. *sabina*, *J. s.* var. *balkanensis* see Adams et al. (2018a) for collection details.

DNA extraction and sequencing

One gram (fresh weight) of the foliage was placed in 20 g of activated silica gel and transported to the lab, thence stored at -20° C until the DNA was extracted. DNA was extracted from juniper leaves by use of a Qiagen mini-plant kit (Qiagen, Valencia, CA) as per manufacturer's instructions. Amplifications were performed in 30 µl reactions using 6 ng of genomic DNA, 1.5 units Epi-Centre Fail-Safe Taq polymerase, 15 µl 2x buffer E (petN, trnD-T, trnL-F, trnS-G) or K (nrDNA) (final concentration: 50 mM KCl, 50 mM Tris-HCl (pH 8.3), 200 µM each dNTP, plus Epi-Centre proprietary enhancers with 1.5 - 3.5

mM $MgCl_2$ according to the buffer used) 1.8 µM each primer. See Adams, Bartel and Price (2009) for the ITS and petN-psbM primers utilized. The primers for trnD-trnT, trnL-trnF and trnS-trnG regions have been previously reported (Adams and Kauffmann, 2010). The PCR reaction was subjected to purification by agarose gel electrophoresis. In each case, the band was excised and purified using a Qiagen QIAquick gel extraction kit (Qiagen, Valencia, CA). The gel purified DNA band with the appropriate sequencing primer was sent to McLab Inc. (San Francisco) for sequencing. 2.31 (Technelysium Pty Ltd.).

Flow cytometric analyses for ploidy level determination

Nuclear DNA amount was assessed by flow cytometry (FC) based on the technique of Bourge et al. (2018) on silica dried leaves of *Juniperus* samples and fresh leaves of *Hordeum vulgare* L. 'Sultan' (2C= 9.81 pg in Garnatje et al. (2004)) used as an internal standard. Approximately, 30 mg of leaves of both the internal standard and *Juniperus* were simultaneously chopped using a razor blade in a plastic Petri dish with 500 µl of cold Gif nuclear-isolation buffer-GNB (Bourge et al. 2018): 30 mM sodium citrate, 45 mM $MgCl_2$, 60 mM MOPS (4-morpholine propane sulphonate, pH 7), and 1% (w/v) polyvinylpyrrolidone 10,000, pH 7.2 containing 0.1% (w/v) Triton X–100, supplemented with 10 mM sodium metabisulphite and RNase (2.5 U/ml). The nuclei suspension was filtered through 50 µm. The nuclei were stained with 100 µg/ml propidium iodide (PI); a specific DNA fluorochrome intercalating dye, and kept at 4°C for 5 min. DNA content of about 3,000 stained nuclei was determined for each sample using the cytometer CytoFLEX S (Beckman Coulter- Life Science United States. Excitation 561 nm, 26 mW; emission through a 610/20 nm band-pass filter). Measurements of each sample were repeated twice. The software CytExpert was used for histogram analyses. The total 2C DNA value was calculated using the linear relationship between the fluorescent signals from stained nuclei of the species and the internal standard, according to the following formula:

2C DNA content/nucleus (pg) = (<u>Sample 2C peak mean / Standard 2C peak mean</u>) x Standard 2C DNA (pg).

RESULTS

Thirteen of *J.* x*pfitzeriana* accessions were tetraploids (4*x*, except Sea Green that was found to be a triploid (3*x*, Table 3). Analysis of three chloroplast regions: petN-psbM, trnS-trnG, and trnL-trnF sequences of the 14 cultivars and all the *Juniperus* taxa with smooth leaf margins in section *Sabina*, revealed that the sequences of all 14 accessions were identical (Fig. 1). Furthermore, the 14 *J.* x*pfitzeriana* accessions cp sequences were identical to that of *J. sabina* var. *balkanensis* (Table 3), and differed by three indels and one SNP from *J. thurifera* and *J. t.* var. *africana* (Fig. 1). Thus, revealing that the tetraploid male (paternal, pollen) parent of *J. xpfitzeriana* was *J. sabina* var. *balkanensis* (or an ancestor with the same chloroplast sequence for petN-psbM, trnS-trnG, and trnL-trnF).

Juniperus chinensis was found to be unacceptable as the male (cp) parent (Table 3) as each of the three cp gene regions were specific for *J. chinensis*. Likewise, *J. chinensis* var. *tsukusiensis* and var. *taiwanensis* (now recognized as *J. tsukusiensis* and *J. tsukusiensis var. taiwanensis,* Adams 2014) are unacceptable, because both contain the *chinensis* type cp, and, interestingly, are diploids (Table 3). *Juniperus chinensis* var. *sargentii* was found to be 4*x*, and contained a different type chloroplast (noted as *sargentii,* Table 3).

Table 2. Collection information for *J.* x*pfitzeriana* at Windsor Gardens, UK.

cultivar name and Adams collection number, All *Juniperus* x*pfitzeriana*	Windsor acc. #	Location in Windsor Gardens	Origin: [1]Krussmann, 1991, [2]The Conifer Manual, Welch, 2012, [3]Conifertreasury.org
'Aurea' *Adams 15474*	1999-6099	HG57 Grayswood 2 (435) 1 1	[1]Mutation of 'Pfitzeriana', similar to type. (= *J. media* var. *pfitzeriana* f. *aurea* van Melle) ex D. Hill Nursery, IL, 1923[1]
'Aurea' *Adams 15418*	na	University of Paris-sud campus	[1]Mutation of 'Pfitzeriana', similar to type. (= *J. media* var. *pfitzeriana* f. *aurea* van Melle) 1923, D. Hill Nursery, IL, Dundee, USA
'Arctic' *Adams 15442*	1999-6077	HG28 Bomer 2 (425) 62 1	[3]1972?, Mitsch Nursery, Aurora, OR, USA D. Hill Nursery, IL, Dundee, USA
'Armstrongii' *Adams 15454*	1999-6075	HG41 Hillier 1 (102) 31 1	[1]low, slow growing 'Pfitzeriana', Dev. 1932, Armstrong Nurseries, Ontario, CA, USA
'Carberry Gold' *Adams 15425*	2001-774	HG16 (423) 15 1	[3]Carbery Nurs., Bournemouth, UK [1] = Old Gold Carberry
'Carberry Gold' *Adams 15463*	1999-6081	HG49 Esveld 1 (97) 2 1	[3]Carbery Nurs., Bournemouth, UK [1] = Old Gold Carberry
'Gold Star' *Adams 15443*	1999-6088	HG28 Bomer 2 (425) 83 2	[1]discovered David Bakker (1961), introduced 1971, Bakker & Sons Nursery, St. Catherine, Ontario, CA
'Golden Saucer' *Adams 15462*	1999-6084	HG44 Bedgebury (88) 34 2	[1]sport of 'Pfitzeriana Aurea', more yellow in winter. 1976, MW Van Nierop, Boskoop, Holland
'Goldenkissen' *Adams 15482*	1999-6086	HG64 Mason (110) 21 5	[3]D M van Delderen, 1983, G. Oltsman Nursery., Ekern, Germany
'Old Gold' *Adams 15453*	1999-6097	HG41 Hillier 1 (102) 21 1	[1]mutation of 'Pfitzeriana Aurea', ex FJ Grootendorst, Holland, 1958.
'Pfitzeriana Prostrate' *Adams 15430*	1999-6102	HG20 (333) 31 1	prostrate sport of *J. Xpfitzeriana* propagated from plant at Windsor
'Saybrook Gold' *Adams 15423*	2001-2555	HG14 (337) 24 4	[2,3]1980, Girard Nursery, Geneva, OH, USA
'Sea Green' *Adams 15436*	1999-6110	HG27 Bomer 1 (91) 30 2	commercial plant, locally available.
'Sea Green' Adams 15604	na	na	Home Depot Inc. nursery, St. George, UT, USA
'Wilhelm Pfitzer' *Adams 15435*	2000-179	HG25 (317) 1 1	[1]male, putative natural hybrid (*J. chinensis x J. sabina*), seeds ex Ho Lan Shan, inner Mongolia, purchased as *J. chinensis pendula,* 1876 by Simon Louis Nursery, Metz, France, and plants sold to the public by Spath Nursery in 1899.

All four samples of *J. sabina* var. *sabina* are diploids (2*x*, Table 3) and contain the var. *sabina* chloroplast, eliminating *J. s.* var. *sabina* as a possible male parent for *J.* x*pfitzeriana.* Having established that the paternal parent of *J.* x*pfitzeriana* is *Juniperus sabina* var. *balkanensis* (4*x*) or an ancestor, it seemed fruitful to investigate the maternal parent of *J.* x*pfitzeriana* by use of the nuclear gene region, nrDNA. Analysis of the 14 cultivars vs. 44 taxa in sect. *Sabina*, smooth leaf junipers revealed that the *J.* x*pfitzeriana* cultivars grouped with *J. chinensis* and *J. s.* var. *balkanensis.* (Fig. 2). Further analysis of nrDNA (1270 bp) revealed 8 variable sites, with 7 of them indicative of hybridization (Table 4). Site 410 was heterozygous in 12 of 14 cultivars, and homozygous in 'Old Gold' and 'Sea Green'. Thus, the nrDNA (ITS) region clearly supports that *J.* x*pfitzeriana* is of hybrid origin. All the 12 *J.* x*pfitzeriana* cultivars had identical nrDNA, except 'Old Gold' and 'Sea Green', that have C and G at 410 and 1075 (Table 4). Interestingly, 'Sea Green' also has a T at site 663, and is a triploid. Sea Green may have been derived from a tetraploid xpfitzer, backcrossed to a diploid *J. chinensis*, giving the triploid Sea Green, based on their having C and G at 410 and 1075.

Table 3. Classification of the 14 *J. xpfitzeriana* (=x*media*) accessions by cp markers. chloroplast types: *balkanensis* = *J. sabina* var. *balkanensis*; *sabina* = *J. sabina var. sabina*; and *chinensis* = *J. chinensis*; *sargentii* = *J. chinensis var. sargentii.*

J. xpfitzeriana (=*xmedia*), unless noted otherwise	ploidy	petN	trnSG	trnLF	chloroplast, ex pollen
15442 Arctic	4*x*	balk	balk	balk	*balkanensis*
15454 Armstrongii	4*x*	balk	balk	balk	*balkanensis*
15418 Aurea, Paris-sud	4*x*	balk	balk	balk	*balkanensis*
15474 Aurea	4*x*	balk	balk	balk	*balkanensis*
15423 Saybrook Gold	4*x*	balk	balk	balk	*balkanensis*
15425 Carberry Gold	4*x*	balk	balk	balk	*balkanensis*
15463 Carberry Gold	4*x*	balk	balk	balk	*balkanensis*
15443 Gold Star	4*x*	balk	balk	balk	*balkanensis*
15462 Golden Saucer	4*x*	balk	balk	balk	*balkanensis*
15482 Goldenkissen	4*x*	balk	balk	balk	*balkanensis*
15430 pfitzeriana prostate	4*x*	balk	balk	balk	*balkanensis*
15435 Wilhelm Pfitzer	4*x*	balk	balk	balk	*balkanensis*
15453 Old Gold	4*x*	balk	balk	balk	*balkanensis*
15436 Sea Green, Windsor	3x	balk	balk	balk	*balkanensis*
15604 Sea Green, Home Depot nursery	3x	balk	balk	balk	*balkanensis?*
Most likely male parent from cp data					
14723 sabina v. balkanensis, Bulg.	4*x*	balk	balk	balk	*balkanensis*
14728 sabina v. balkanensis, Greece	4*x*	balk	balk	balk	*balkanensis*
Unacceptable as male (pollen) parent					
8535 chinensis, Japan, Kaizuka?	4*x*	chin	chin	chin	*chinensis*
8536 chinensis, Japan, Kaizuka?	4*x*	chin	chin	chin	*chinensis*
9061 chin. v. taiwanensis, Taiwan (=tsukusiensis var. taiwanensis)	2*x*	chin	chin	chin	*chinensis*
8805 chin, v. tsukusiensis, Japan (= tsukusiensis v. tsukusiensis)	2*x*	chin	chin	chin	*chinensis*
8688 chinensis v. sargentii, Japan	4*x*	sarg	sarg	sarg	*sargentii*
14316 sabina v. sabina, Azerbaijan	2*x*	sab	sab	sab	*sabina*
7614 sabina v. sabina, Switzerland	2*x*	sab	sab	sab	*sabina*
7573 sabina v. sabina, Pyrenees	2*x*	sab	sab	sab	*sabina*
7811 sabina v. sabina, Kazakhstan	2*x*	sab	sab	sab	*sabina*

Examining the variable sites (i.e., hybrid indicating sites, or hybrid sites) of *J. chinensis, J. chinensis* var. *tsukusiensis* (now *J. tsukusiensis* var. *tsukusiensis*), *J. c.* var. *taiwanensis (now J. tsukusiensis* var. *taiwanensis*), and *J. sargentii*, revealed that all of these taxa (except *J. c.* var. *sargentii*) have the correct sequences at the hybrid sites to be the maternal parent of *J.* x*pfitzeriana* (Table 4). *Juniperus sargentii* is not likely the maternal parent because it has 3 non-matching bases at sites 663, 985, 1075 (Table 4).

Another factor to consider in the potential maternal parent of *J.* x*pfitzeriana* is the ploidy level. Notice that *J. chinensis* (samples from Japan) is a tetraploid (4x), whereas *J. c.* var. *tsukusiensis* and *J. c.* var. *taiwanensis* are both diploids (2x, Table 4). Thus, *J. chinensis* (4x) seems more probable as the maternal parent of *J.* x*pfitzeriana.*

Table 4. nrDNA (ITS) variable sites in *J.* x*pfitzeriana* (=x*media*) (Windsor Gardens), *J. chinensis*, and *J. sabina*. K=G/T; S=C/G; Y=C/T; M=A/C; W=A/T; R=A/G. chloroplast types: *balkanensis* = *J. sabina* var. *balkanensis*/ *J. thurifera*; *sabina* = *J. sabina var. sabina*; and *chinensis* = *J. chinensis.*

taxa: *J.* x*pfitzeriana* (=x*media*), unless noted otherwise	ploidy	212 K	410 S	663 Y	985 Y	995 M	1033 K	1075 W	1139 R	ITS classif. hybrid?	chloroplast, ex. pollen
15442 Arctic	4*x*	G/T	C/G	C/T	C/T	A/C	G/T	A/T	A/G	chin x sab	*balkanensis*
15454 Armstrongii	4*x*	G/T	C/G	C/T	C/T	A/C	G/T	A/T	A/G	chin x sab	*balkanensis*
15418 Aurea, Paris-sud	4*x*	G/T	C/G	C/T	C/T	A/C	G/T	A/T	A/G	chin x sab	*balkanensis*
15474 Aurea	4*x*	G/T	C/G	C/T	C/T	A/C	G/T	A/T	A/G	chin x sab	*balkanensis*
15423 Saybrook Gold	4*x*	G/T	C/G	C/T	C/T	A/C	G/T	A/T	A/G	chin x sab	*balkanensis*
15425 Carberry Gold	4*x*	G/T	C/G	C/T	C/T	A/C	G/T	A/T	A/G	chin x sab	*balkanensis*
15463 Carberry Gold	4*x*	G/T	C/G	C/T	C/T	A/C	G/T	A/T	A/G	chin x sab	*balkanensis*
15443 Gold Star	4*x*	G/T	C/G	C/T	C/T	A/C	G/T	A/T	A/G	chin x sab	*balkanensis*
15462 Golden Saucer	4*x*	G/T	C/G	C/T	C/T	A/C	G/T	A/T	A/G	chin x sab	*balkanensis*
15482 Goldenkissen	4*x*	G/T	C/G	C/T	C/T	A/C	G/T	A/T	A/G	chin x sab	*balkanensis*
15430 pfitzeriana prostate	4*x*	G/T	C/G	C/T	C/T	A/C	G/T	A/T	A/G	chin x sab	*balkanensis*
15435 Wilhelm Pfitzer	4*x*	G/T	C/G	C/T	C/T	A/C	G/T	A/T	A/G	chin x sab	*balkanensis*
15453 Old Gold	4*x*	G/T	**C**	C/T	C/T	A/C	G/T	A/T	**G**	chin x sab*	*balkanensis*
15436 Sea Green, Windsor	3x	G/T	**C**	**T**	C/T	A/C	G/T	A/T	**G**	chin x sab*	*balkanensis*
15604 Sea Green Home Depot	3x	G/T	**C**	**T**	C/T	A/C	G/T	A/T	**G**	chin x sab*	*balkanensis?*
8535 chinensis, Japan	4*x*	T	C	C	C	C	G	A	G	*chinensis*	*chinensis*
8536 chinensis, Japan	4*x*	T	C	C	C	C	G	A	G	*chinensis*	*chinensis*
9061 chin. v. taiwanensis, Taiwan (=tsukusiensis var. taiwanensis)	2*x*	T	C	C	C	C	G	A	G	*chinensis*	*chinensis*
8805 chin, v. tsukusiensis, Japan (= tsukusiensis v. tsukusiensis)	2*x*	T	C	C	C	C	G	A	G	*chinensis*	*chinensis*
8688 chinensis v. sargentii, Japan	4*x*	T	C	T	T	C	G	T	G	chin sarg.	*sargentii*
sabina Type 2 ITS											
14723 sabina v. balkanensis, Bulg.	4*x*	G	C	T	T	A	T	T	G	*sab. v. balk*	*balkanensis*
14316 sabina v. sabina, Azerbaijan	2*x*	G	C	T	T	A	T	T	G	*sabina*	*sabina*
7614 sabina v. sabina, Switzerland	2*x*	G	C	T	T	A	T	T	G	*sabina*	*sabina*
sabina Type 1 ITS:											
14728 sabina v. balkanensis, Greece	4*x*	G	C	T	T	A	G	T	G	*balkanensis*	*balkanensis*
7573 sabina v. sabina, Pyrenees	2*x*	G	C	T	T	C	G	T	G	*sabina*	*sabina*
7811 sabina v. sabina, Kazakhstan	2*x*	G	C	T	T	A	G	T	G	*sabina*	*sabina*
Most probable male (pollen) parent genotype	4*x*	G	C	T	T	A	T	T	G	*balkanensis* Type 2 ITS	*balkanensis*
male parent: pollen, with balk cp. 14723 sabina v. balkanensis, Bulg.	4*x*	G	C	T	T	A	T	T	G	*balkanensis* Type 2 ITS	*balkanensis*
typical x*pfitzeriana*, cf 15442, above	4*x*	G/T	C/G	C/T	C/T	A/C	G/T	A/T	A/G	chin x balk	*balkanensis*
Most probable female parent genotype	**4*x***	T	G	C	C	C	G	A	A	*chinensis*	*chinensis*
female parent: 8535 chinensis, Japan	4*x*	T	C	C	C	C	G	A	G	chinensis	*chinensis*

Variable sites located at: 212, xGGCCAAGC; 410, xGTTGAGAT; 663, xTCTTCGTC; 985, xGCCCTCCC; 995, xGCGAGGAG; 1033, xGCGGTCGG; 1075, xCGCGACGA; 1139, xGAACTTTG.

Although we have established that the paternal parent is *J. sabina* var. *balkanensis* (or an ancestor). There is an 8 site polymorphism in the nrDNA of *J. sabina*, which Adams et al. (2018a, b) referred to as Type 1 and Type 2. Examination of nrDNA (ITS) Type 1 and Type 2 variation (Table 5) shows that there is no variation in the 14 *J.* x*pfitzeriana* cultivars. *Juniperus chinensis* (8535, 8536) has a slightly different Type 2 pattern with a C in 995 and a G in 1036 that perfectly complements the paternal *balkanensis* ITS Type 2 pattern to make the observed A, C, G, C, T, A/C, G, G/T pattern of *J.* x*pfitzeriana.*

Table 5. nrDNA (ITS) Type 1 and Type 2 nrDNA at 8 variable sites in *J.* x*pfitzeriana* (=x*media*) (Windsor Gardens), *J. chinensis*, and *J. sabina*. [1]Eight polymorphic sites are 350(R), 391(S), 432(R), 604(M), 745(Y), 995(M), 1036(R), 1037(K).

taxa: *J. xpfitzeriana* (=x*media*), unless noted otherwise	ploidy	[1]350	391	432	604	745	995	1036	1037	ITS Type	cp male parent
Type 1 nrDNA (ITS) pattern		G	G	A	C	C	C	A	G	1	
Type 2 nrDNA (ITS) pattern		A	C	G	A	T	A	G	T	2	
15442 Arctic	4*x*	A	C	G	A	T	A/C	G	G/T	2	balkanensis
15454 Armstrongii	4*x*	A	C	G	A	T	A/C	G	G/T	2	balkanensis
15418 Aurea, Paris-sud	4*x*	A	C	G	A	T	A/C	G	G/T	2	balkanensis
15474 Aurea	4*x*	A	C	G	A	T	A/C	G	G/T	2	balkanensis
15423 Saybrook Gold	4*x*	A	C	G	A	T	A/C	G	G/T	2	balkanensis
15425 Carberry Gold	4*x*	A	C	G	A	T	A/C	G	G/T	2	balkanensis
15463 Carberry Gold	4*x*	A	C	G	A	T	A/C	G	G/T	2	balkanensis
15443 Gold Star	4*x*	A	C	G	A	T	A/C	G	G/T	2	balkanensis
15462 Golden Saucer	4*x*	A	C	G	A	T	A/C	G	G/T	2	balkanensis
15482 Goldenkissen	4*x*	A	C	G	A	T	A/C	G	G/T	2	balkanensis
15430 pfitzeriana prostate	4*x*	A	C	G	A	T	A/C	G	G/T	2	balkanensis
15435 Wilhelm Pfitzer	4*x*	A	C	G	A	T	A/C	G	G/T	2	balkanensis
15453 Old Gold	4*x*	A	C	G	A	T	A/C	G	G/T	2	balkanensis
15436 Sea Green, Windsor	3x	A	C	G	A	T	A/C	G	G/T	2	balkanensis
15604 Sea Green, Windsor	3x	A	C	G	A	T	A/C	G	G/T	2	balkanensis
8535 chinensis, Japan	4*x*	A	C	G	A	T	C	G	G	2*	maternal chinensis
8536 chinensis, Japan	4*x*	A	C	G	A	T	C	G	G	2*	
9061 chin. v. taiwanensis, Taiwan (=tsukusiensis var. taiwanensis)	2*x*	A	C	G	A	T	C	G	G	2*	
8805 chin, v. tsukusiensis, Japan (= tsukusiensis v. tsukusiensis)	2*x*	A	C	G	A	T	C	G	G	2*	
8688 chinensis v. sargentii, Japan	4*x*	A	C	G	A	T	C	G	G	2*	
14723 sabina v. balkanensis, Bulg.	4*x*	A	C	G	A	T	A	G	T	2	paternal balkanensis
14316 sabina v. sabina, Azerbaijan	2*x*	A	C	G	A	T	A	G	T	2	
7614 sabina v. sabina, Switzerland	2*x*	A	C	G	A	T	A	G	T	2	
14728 sabina v. balkanensis, Greece	4*x*	G	G	A	C	C	C	A	G	1	
7573 sabina v. sabina, Pyrenees	2*x*	G	G	A	C	C	C	A	G	1	
7811 sabina v. sabina, Kazakhstan	2*x*	G	G	A	C	C	C	A	G	1	

[1]Eight polymorphic sites (1-8): polymorphic sites are 350(R), 391(S), 432(R), 604(M), 745(Y), 995(M), 1036(R), 1037(K). 350 xTGTCGGAG; 391 xGAGGTCCG; 432 xTCGTGTGC; 604 CGACAAGAx; 745(105) xCCAAAAGA; 995(333) xGCGAGGAG; 1036(392) xNGCGGTCGG;1037 xGCGGTCGG

A caveat to the aforementioned analysis is that the *J. chinensis* (8535, 8536) from Japan appear to be cv. Kaizuka, with spiral, twisted branches. Krussmann (1991) noted that 'Kaizuka' or 'Hollywood' juniper came from the Yokohama Nursey in the 1920s to the USA. Although I (RPA) collected from trees growing in a 'natural appearing' site; the site may have been planted in a 'randomly natural' manner. *Juniperus chinensis* is a very widely cultivated in China and Farjon notes in his contribution to the ICUN Red List (https://www.iucnredlist.org/species/42227/2962948#habitat-ecology):

"In a few localities this widespread species forms groves of tall trees (e.g. in S Gansu), or it is mixed with pines and deciduous angiosperms at canopy level. It is much more common, under conditions largely determined by man's agricultural practices, in secondary vegetation, on open, rocky slopes. The altitudinal range is (100-)1,400-2,400(-2,700) m a.s.l. Widespread planting and subsequent establishment in areas where it was not originally native have made it difficult to establish it original habitat and types of vegetation."

In a recent communication with Kangshan Mao (Chengdu), he wrote that there may be a few isolated trees in the mountains of southern Gansu, and that his students will undertake a survey/ collection trip in the summer of 2019. Collecting samples of *J.* x*pfitzeriana* plants, *J. chinensis* and *J. sabina* in the Ho Lan (Helan) Mountains (Shan) seems promising (research in progress).

ACKNOWLEDGEMENTS

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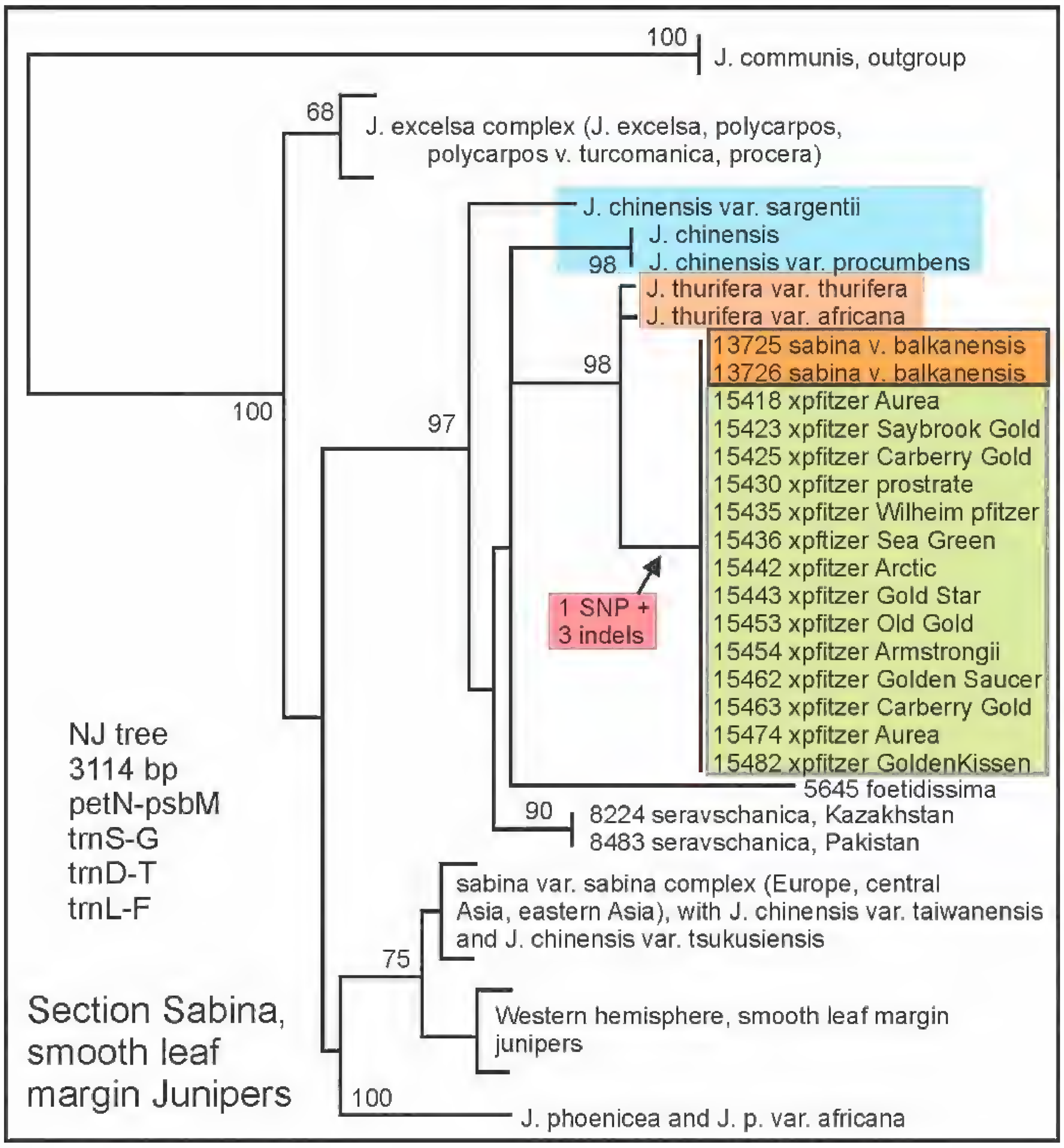

Figure 1. Chloroplast tree for *Juniperus*, sect. *Sabina*, smooth leaf margined junipers, based on four chloroplast gene regions, 3114 bp: petN-psbM, trnS-trnG, trnD-trnT, and trnL-trnF. Numbers at branch points are posterior probabilities as percent. Probabilities below 68 are not shown. Notice that *J. thurifera and J. thurifera var. africana* differ by only 1 SNP and 3 indels from xpfitzer (*J.* x*pfitzeriana*) cultivars. There are no sequence differences among the xpfitzer (*J.* x*pfitzeriana*) cultivars, nor with *J. sabina* v. *balkanensis* (inside yellow and orange boxes).

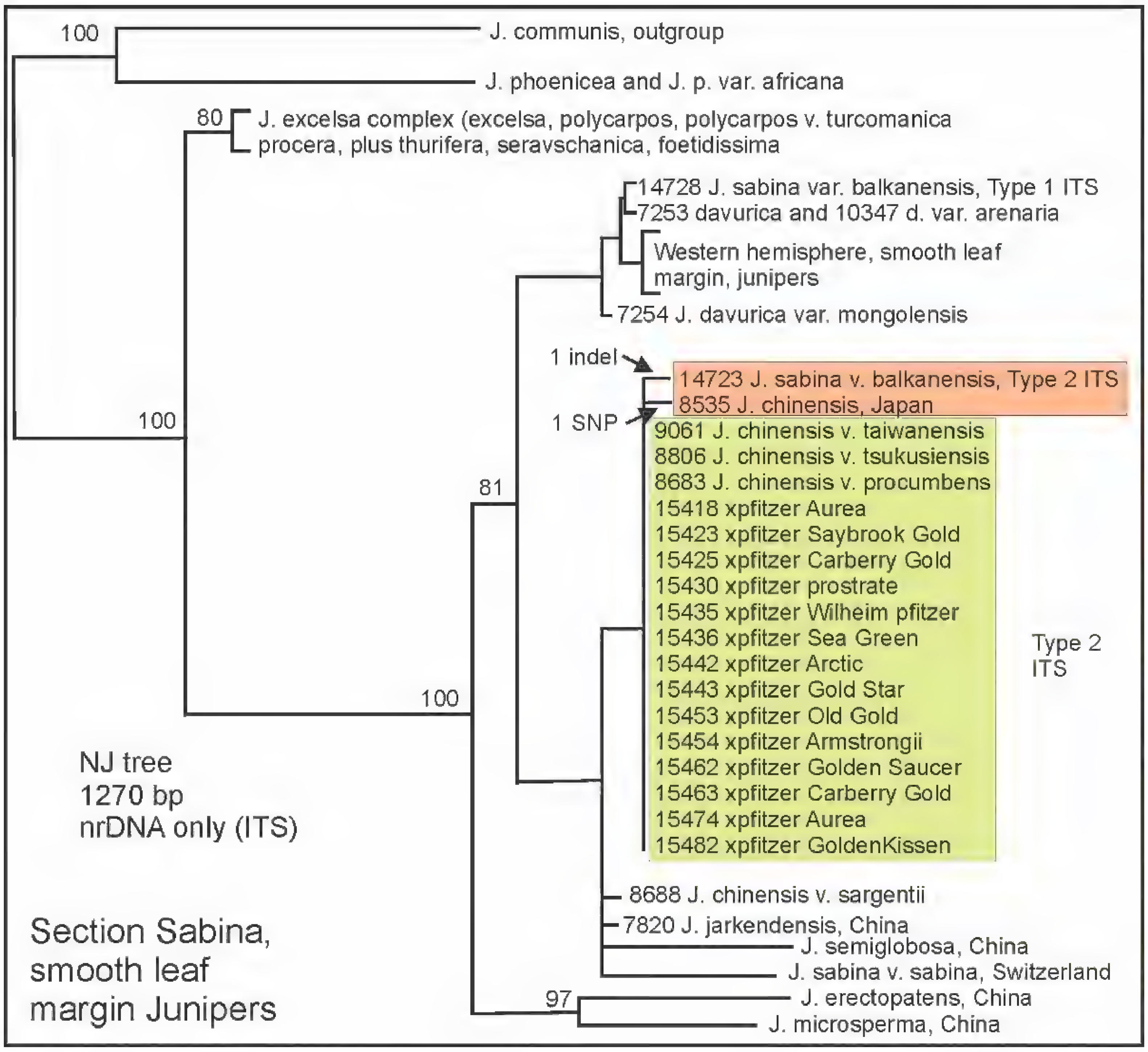

Figure 2. nrDNA (ITS) tree for *Juniperus*, sect. *Sabina*, smooth leaf margined junipers, based nrDNA (ITS), 1270 bp. Numbers at branch points are posterior probabilities as percent. Probabilities below 68 are not shown. Notice that 14723 *J. sabina* var. *balkanensis* differs by only 1 indel from xpfitzer cultivars and 8535 *J. chinensis*, Japan differs by only 1 SNP from the xpfitzer cultivars. There are no sequence differences among the xpfitzer (*J. xpfitzeriana*) cultivars, nor with *J. chinensis* v. *procumbens*, v. *taiwanensis* or v. tsukusiensis, except for the heterozygous sites in the xpfitzer cultivars (inside yellow box).

Rodmanochytrium is a new genus of chitinophylic chytrids (_Chytridiales_)

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ABSTRACT

From biodiversity surveys of aquatic habitats using chitin and pollen baits with samples collected in Argentina and the United States, we found a clade of eight chytrid strains with morphologies distinct from other described chytrids. After bringing these strains into axenic culture, we studied their thallus development and molecular phylogeny. Thalli produce operculate sporangia of variable shapes, ranging from spherical, pyriform to multilobed, and with rhizoidal systems exhibiting a sub-sporangial tube or swelling. Surfaces of sporangia could be smooth or ornamented. Resting spores are endobiotic when strains are grown on sweet gum pollen. Molecular phylogeny of 28S rDNA sequences place these eight strains in the *Chytriomycetaceae*. Based on thallus morphology, molecular phylogeny and zoospore ultrastructural features, we classify these eight strains as a new genus, *Rodmanochytrium*, containing two species, *R. sphaericum* and *R. pyriforme*. Published on-line www.phytologia.org *Phytologia 101(3): 175-187 (Sept 21, 2019)*. ISSN 030319430.

KEY WORDS: *Rodmanochytrium* M.J. Powell & Letcher, *gen. nov*., *Rodmanochytrium sphaericum* M.J. Powell & Letcher, *sp. nov., Rodmanochytrium pyriforme* M.J. Powell & Letcher, *sp. nov*., chitin, chytrid, *Chytridiomycota,* morphology, phylogeny, pollen, systematics

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Recent explorations of aquatic habitats for chytrids have revealed that there is much undescribed diversity within the *Chytridiales* (Lefèvre et al., 2012; Leshem et al., 2016; Letcher and Powell, 2018; Letcher et al., 2014a, 2014b, 2018; Picard et al., 2009; Powell et al., 2013, 2018, 2019; Seto and Degawa, 2018; Seto et al., 2017; Vélez et al., 2013). From surveys of pollen and chitin-inhabiting chytrids, we isolated eight unidentified strains. Several of these unidentified isolates have been included in earlier molecular phylogenetic studies but never described. In a broad survey of *Chytridiomycetes*, strain WB 235A placed in the *Chytridiales* (James et al., 2006). In broad surveys of *Chytridiales*, several of the strains placed sister of the type species of *Chytriomyces, C. hyalinus,* in the *Chytriomycetaceae* (strains ARG 12, ARG 39, WB 235A; Davis et al., 2015; Letcher and Powell, 2014; Letcher et al., 2014a, 2018; Powell et al., 2018). Letcher and Powell (2014) determined that zoospore ultrastructure of strains WB235A, ARG12, and ARG39 was a Group 1-type as Barr (1980) first characterized. These results support classification in *Chytriomycetaceae* based on the following zoospore ultrastructural character states: i. nucleus partially inserted into ribosomal aggregation; ii. kinetosome-associated structure (KAS) consists of stacked plates; iii. microtubular root includes multiple microtubules in a bundle; iv. microbody-lipid globule (MLC) complex cisterna fenestrated; v. flagellar plug bi-concave and thin (~90nm); and vi. paracrystalline inclusion large (vol. > 0.03 μm^3) (Letcher and Powell, 2014). Because of the distinct morphology and phylogenetic placement of these chytrid strains, we characterize a new genus, *Rodmanochytrium*, comprised of two species.

MATERIALS AND METHODS

Locations where strains used in this study were collected are listed in Table 1. Pine pollen, sweet gum pollen, dragonfly wings, and purified shrimp exoskeleton chitin strips were added as baits to aquatic samples. Strains were isolated and cultured on PmTG agar as described (Powell et al., 2019). Strains growing on natural substrates or nutrient media were observed with bright field and Nomarski interference contrast optics (Powell et al., 2019). Strains growing on chitin were prepared for scanning electron microscopy (SEM) and observed following the protocol in Picard et al. (2009).

DNA was extracted, purified and the partial large subunit RNA gene (28S rDNA) amplified and sequenced as described previously (Powell et al., 2019). Eight new gene sequences, which we produced (noted in Table 1 with *), along with sequences downloaded from GenBank (Table 1), were aligned with Clustal X and manually adjusted in BioEdit (Powell et al., 2019). Phylogenetic analyses included 35 strains of in-group taxa rooted with strain JEL 222 *Rhizophydium globosum* as the outgroup. Because of earlier phylogenetic placements, our analysis included 26 representatives of the morpho-species *Chytriomyces hyalinus*. *Rhopalophlyctis sarcoptoides* is included because of its sister position to *C. hyalinus*. Maximum parsimony (MP) trees were generated, maximum likelihood (ML) phylogenies constructed, and bootstrap support values calculated as described (Powell et al., 2019; Vélez et al., 2011). Sequence similarities among strains were determined with pairwise alignment in BioEdit (Hall, 1999).

RESULTS

For MP analysis, the dataset had 805 characters, of which 131 were parsimony informative. Of the 1005 trees derived from PAUPRat, all were equal in length (L = 228 steps) and were used to construct a single, strict consensus tree. For ML analysis, Modeltest selected HKY as the most appropriate model of nucleotide substitutions. The topologies of cladograms from MP and ML (-lnL = 956.58) analyses were identical, with similar or equal support values. Minimum value for bootstrap support was set at 70%. Figure 1 shows the strict consensus tree from MP analysis, with ML/MP support values at nodes. Two major lineages (lineages A and B) were resolved.

Lineage A with $\geq$ 99% bootstrap support included the eight unidentified strains in two sub-clades (A1, A2). Each of the sub-clades in lineage A is well supported, A1 with $\geq$ 98% bootstrap support and A2 with 100% support. Interestingly in lineage A2, the two strains from Argentina show stronger relationships with strains from Alabama than they do with each other (Fig. 1). Within sub-clade A1, 28S rDNA sequence similarity is 98.0%; within subclade A2, sequence similarities range from 97.9–100%. Sub-clades A1 and A2 are divergent from each other, with strain MP 72 (sub-clade A1) having 93% sequence similarity with strain MP 41 (sub-clade A2). Thus, we consider that each of the two sub-clades in lineage A represents a species.

Lineage A is sister of lineage B ($\geq$ 79% bootstrap support), which includes representatives of two other chitinophylic species (each the type species for their genus), *Rhopalophlyctis sarcoptoides* and the morpho-species *Chytriomyces hyalinus* (Fig. 1). The eight unidentified strains in lineage A are phylogenetically distinct from these other chitinophylic species in lineage B. For example, MP 72 in sub-clade A1 has only 88% sequence similarity with JEL 794 *Rhopalophlyctis sarcoptoides*. Thus, we consider members of lineage A as a distinct genus from *Rhopalophlyctis* and *Chytriomyces*.

TAXONOMY

Rodmanochytrium M.J. Powell & Letcher, ***gen. nov.***

MycoBank no.: 830008

Typification: *Rodmanochytrium pyriforme* M.J. Powell & Letcher, sp. nov. (TYPE SPECIES).

Etymology: Named in honor of Dr. James E. Rodman, former program director in the Division of Environmental Biology at the US National Science Foundation, recognizing his implementation of the Partnerships for Enhancing Expertise in Taxonomy (PEET) program. This program targeted support for poorly studied groups of organisms, including chytrids, resulting in new species discovery, updated monographs, electronic access to data and a new generation of systematists trained.

Description: Sporangium: monocentric, epibiotic; pyriform, clavate, oval, or spherical, rarely lobed; surface smooth, hirsute, reticulate, rugose or finely granular; Rhizoid: rhizoidal axis emerges as a single tube from the sporangium, slightly swollen near the sporangium, dividing into branches near the sporangium; at maturity sub-sporangial portion tubular or swollen, rhizoidal system finely branched and pointed at the tips; dome-shaped septum separates rhizoids and sporangium. Zoospore Discharge: single, apical to lateral, relatively small operculate discharge pore; gelatinous plug forms below the operculum prior to discharge; operculum detached or hinged to side of pore after discharge; vesicular discharge with zoospores formed in sporangium, released as a quiescent mass, swarming and then swimming away. Zoospore: posteriorly uniflagellate, elongate, typically with a single lipid globule. Resting spore: typically endobiotic, globose or ovoid; thick walled with numerous lipid globules; germination not observed. Group 1-type zoospore. Monophyletic in *Chytriomycetaceae*.

Rodmanochytrium pyriforme M.J. Powell & Letcher, *sp. nov*.

MycoBank no.: 830009 Figs. 2, 3

Typification: UNITED STATES, Alabama, Washington County, Wagarville, Sullivan Lane, 31.4730583333, -88.037250. From a water and moist soil sample collected from a crawfish pond spring 2010 by J. K. Atchison and baited with chitin; strain MP 72 isolated by M. J. Powell, HOLOTYPE Fig. 2G, this publication.

Ex-Type Strain: MP 72 deposited in CZEUM (University of Michigan).

Etymology: The specific epithet refers to the predominant pyriform shape of the sporangium.

Description: Sporangium: predominantly pyriform, occasionally clavate, oval or spherical, rarely lobed; width at widest part typically 25-40 µm, sometimes up to 65 µm; surface smooth, or rugose, often hirsute over the upper, expanded portion of the sporangium. Rhizoids: emergent from base of encysted zoospore as a slightly swollen tube; branches form near the sporangium and branch dichotomously; at maturity finely branched with pointed tips; dome-shaped septum separating rhizoids and sporangium. Zoospore Discharge: from a single, apical, sub-apical or lateral operculate discharge pore, which is relatively small; gelatinous plug forms beneath the operculum prior to zoospore discharge; operculum remains hinged to side of discharge pore or detaches; edge of discharge pore recurved; zoospores cleaved within sporangium, released as a motionless mass, after a quiescent period they begin to swarm and then swim away. Zoospore: elongate 4.5-5 µm long, typically with a single lipid globule. Resting spores: typically endobiotic; spherical or oval, 10-20 µm wide; containing numerous lipid globules; germination not observed. Saprotrophic on pollen and chitinous substrates.

Additional specimens examined: UNITED STATES, Alabama, Tuscaloosa County, Coker, Lake Lurleen. From an aquatic sample containing submerged vegetative matter, collected 29 February 2005 by W. H. Blackwell at the water's edge under an alder tree and baited with dragonfly wings; strain WB 235A isolated by M. J. Powell.

GenBank sequences of ex-type strain MP 72: MK543214 (28S rDNA).

Comments: Zoospores encyst and produce a long germ tube (Fig. 2A). The zoospore cyst enlarges into the incipient sporangium, which is typically pyriform (Figs. 2C, 3A); a small swelling forms on the rhizoidal axis at the base of the sporangium, and the rhizoidal axis branches (Figs. 2B, C, 2F). The enlarging sporangium is typically pyriform (see Kirk et al., 2008 for description of shapes), broad at the top and tapering toward the base (Figs. 2F-I, 3A, B, F-H). Occasionally the sporangium is oval, with a small cup-shaped base (Fig. 2D), or clavate (Fig. 2E). Sporangia are rarely irregular or lobed (Fig. 3D). Sporangial surfaces can be smooth (Fig. 3E), rugose (Fig. 3C, D) or hirsute, with hair-like structures covering the upper, expanded portion of the sporangium (Fig. 3E-I). The sub-sporangial rhizoid on mature sporangia may be branched with a slightly elongate (Figs. 2G, 3B) or spherical (Figs. 2L, 3C)

swelling joined to the tapered based of the sporangium through a short tube (Fig. 3F). A dome-shaped septum separates the sporangium from the rhizoidal system (Fig. 2L). Prior to zoospore discharge a gelatinous plug forms below the operculum (Fig. 2H). Zoospore discharge is vesicular (Fig. 2I), and the single, operculate discharge pore is apical (Fig. 2H, J), sub-apical (Figs. 2K) or lateral (Fig. 2I). The operculum remains attached to the side of the discharge pore (Fig. 2J) or becomes detached (Fig. 2K). The edge of the discharge pore is typically recurved (Fig. 2K). Spherical (Fig. 2M) or oval (Fig. 2N) resting spores are endobiotic in pollen. A tube connects the zoospore cyst at the surface of the pollen grain with the resting spore inside the pollen grain (Fig. 2M). Rather than enlarging into a sporangium, these zoospore cysts expel their contents into the pollen grain through the connecting tube in the process of resting spore formation (Fig. 2M).

Rodmanochytrium sphaericum M.J. Powell & Letcher, *sp. nov.*

MycoBank no.: 830010 Figs. 4, 5

Typification: UNITED STATES, Alabama, Tuscaloosa. From an aquatic sample containing *Myriophyllum* roots collected 5 June 2009 from a stream adjacent to 3621 Greensboro, Ave. and baited with chitin; strain MP 41 isolated by M. J. Powell, HOLOTYPE Fig. 4E, this publication.

Ex-Type Strain: MP 41 deposited in CZEUM (University of Michigan).

Etymology: The specific epithet refers to the predominant spherical shape of the sporangium.

Description: Sporangium: predominantly spherical, occasionally oval or pyriform, rarely lobed; width typically 20-55 µm, sometimes 70-110 µm; surface smooth, reticulate, or finely granular. Rhizoids: emerge from the encysted zoospore as a single long germ tube with a slight swelling near the incipient sporangium; rhizoids branch near the sporangium and become bushy; at maturity rhizoids near sporangium are stout with dichotomous branches tapering to a pointed tip. Zoospore Discharge: from a single, operculate discharge pore, which is relatively small and typically apical; gelatinous plug forms beneath the operculum prior to zoospore discharge; operculum remains hinged to side of discharge pore or detaches; edge of discharge pore not recurved; zoospores cleaved within sporangium, released as a motionless mass, after a quiescent period zoospores begin to swarm and then swim away. Zoospore: elongate 4.5-5 µm long, typically with a single lipid globule. Resting Spores: not observed. Saprotrophic on pollen and chitinous substrates.

Additional specimens examined: UNITED STATES, Alabama, Wheeler National Wildlife Refuge. From an aquatic sample collected 9 August 2009 by B. Swan and baited with pollen; strains MP 59, MP 60, MP 61 isolated by M. J. Powell. ARGENTINA, Buenos Aires Province, Partido de Escobar, Paraná River floodplain. From an aquatic sample collected January 2005 from a rain pool and baited with pollen; strain ARG 12 isolated by C. G. Vélez. ARGENTINA, Corrientes Province. From an aquatic sample collected June 2006 from Chañar Stream and baited with pollen; strain ARG 39 isolated by C. G. Vélez.

GenBank sequences of ex-type strain MP 41: JX905522 (28S rDNA).

Comments: Zoospores encyst and produce an elongate germ tube (Fig. 4A). The portion of the germ tube near the encysted zoospore is slightly enlarged (Fig. 4B) and then branches, commonly dichotomously (Fig. 4C), or elongates into a trunk-like rhizoidal axis (Fig. 4D). Sporangia are typically spherical (Figs. 4E, G, H, J, K, 5A-D), but are sometimes oval (Fig. 4I) or broadly pyriform (Fig. 4F). The sporangial surface is variable, smooth (Fig. 5E), finely granular (Fig. 5A, B), or reticulate (Fig. 5B, C, D). The cytoplasm appears zoned in maturing sporangia; the central region is brown and granular and the periphery is yellowish and homogeneous (Fig. 4I). The branches of the primary rhizoidal axis often extend laterally on nutrient media (Fig. 4E), pollen (Fig. 4G) and chitin (Fig. 5A). On pollen the thallus can be epibiotic or interbiotic (Fig. 4G). Branches extending from the primary rhizoidal axis are stout near the sporangium (Fig. 4E, G-I), but continue dividing into fine branches with pointed tips (Fig. 4H, I). Zoospore discharge is vesicular (Fig. 4J), typically through an apical operculate discharge pore (Figs. 4J, K, 5D, E). The operculum remains hinged to the side of the discharge pore after zoospore release (Figs. 4K, 5E) or detaches.

DISCUSSION

Our results confirm earlier molecular phylogenetic studies of several of these unidentified strains (strains ARG 12, ARG 39, WB 235A) suggesting that they were members of the *Chytriomycetaceae* in the *Chytridiales* (Davis et al., 2015; Letcher and Powell, 2014; Letcher et al., 2014a, 2018). Previous ultrastructural analysis of zoospores of these isolates demonstrated characteristics consistent with other members of the *Chytriomycetaceae, especially of the Chytriomyces hyalinus clade (Letcher and Powell, 2014).*

Although thallus morphology in both of the two species of *Rodmanochytrium* is variable, typical of morphological plasticity found in other chytrids (Powell and Koch, 1977), the two species can be distinguished morphologically. Both species are rarely lobed, but this feature has been found as a variable characteristic in other related species, such as in *Chytriomyces hyalinus* shown to have crowding-induced irregularly-shaped sporangia *(Roane and Paterson, 1974).* The predominantly spherical shape of sporangia distinguishes *R. sphaericum* from *R. pyriforme* with predominantly pyriform sporangia, although *R. sphaericum* sporangia can sometimes be oval and rarely pyriform. The rhizoidal axes near the sporangium are stouter in *R. sphaericum* thalli than in *R. pyriforme* thalli. The main rhizoidal axis of *R. sphaericum* is typically a single short tube with a greater abundance of branches near the sporangium than in *R. pyriforme*. Also, *R. sphaericum* can produce sporangia in a larger size range than found with *R. pyriforme*. The edges of discharge pores on sporangia of *R. sphaericum* are even while those of *R. pyriforme* are recurved. Sporangial surfaces of both species may be smooth at thallus maturity, but some sporangia of *R. sphaericum* have reticulate surfaces while some sporangia of *R. pyriforme* have hirsute surfaces.

Chytrids producing sporangia with pyriform shapes are common, but *Rodmanochytrium* is distinct from these other described species. Unlike *Rodmanochytrium,* which is operculate, many of these are among the inoperculate chytrids, including *Podochytrium chitinophilum* (Willoughby, 1961), *Rhizophydium clavatum, R. clinopus, R. collapsum, R. obpyriformis, R. pirifomis, R. utriculare* and *Phlyctochytrium indicum* (Karling, 1977; Sparrow, 1960). The operculate chytrids with pyriform sporangial shapes, such as *Chytridium cejpii, C. lagenula, C. lecythii, C. pyriforme, C. rhizophydi*, *C. sexuale*, *C. surirellae, C. versatile,* are parasitic (Karling, 1977; Sparrow, 1960), while *Rodmanochytrium* is saprotrophic.

In our phylogeny, *Rodmanochytrium* is sister of a clade including *Chytriomyces hyalinus* and *Rhopalophlyctis sacroptoides*, both operculate chytrids with vesicular zoospore discharge and the ability to grow on chitin-containing substrates. *Rodmanochytrium* is distinct from these other two genera. The endobiotic resting spore of *Rodmanochytrium* distinguishes it from *Chytriomyces*, which has epibiotic resting spores (Karling, 1945), although this can be a variable character (Roane and Paterson, 1974). In addition, *Rodmanochytrium* is not parasitic, as are members of *Chytriomyces* with pyriform shaped sporangia, including *Chytriomyces laevis, C. verrocosus* and *C. willoughbyi* (Karling, 1960, 1968, 1987). The phylogenetic placement of *Rodmanochytrium* further demonstrates that it is not a member of the genus *Chytriomyces* because *C. hyalinus* is the type species of this genus (Letcher and Powell, 2002). The pyriform shaped sporangium in *Rodmanochytrium* is reminiscent of the *Rhopalophlyctis* thallus (Karling, 1945) but lacks the basal sterile cell found in the latter (Karling, 1945). Molecular sequence divergence of 28S rDNA between *Rodmanochytrium* and *Rhopalophlyctis* supports distinct genera (88% sequence similarity between strains MP 72 versus JEL 794).

Because chytrid thalli are relatively simple, morphological characters are often convergent because of limited ways to respond morphologically to natural selection. Similarly, morphological plasticity makes identification of chytrids based on thallus morphology alone difficult (Powell and Koch, 1977). This study demonstrates again that, in addition to thallus morphology, consideration of zoospore ultrastructural

characters and molecular phylogenetic placement is necessary to adequately characterize new taxa of chytrids (Letcher and Powell, 2014).

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Table 1. Strains used for phylogenetic analysis				
Strain	GenBank Number	Location of Collection	Habitat	Substrate
		Ingroup		
ARG 12	JX905504	ARG, Buenos Aires Province, Partido de Escobar	aquatic	pollen
ARG 37	JX905505	ARG, Rìo de la Plata River, Buenos Aires City	aquatic	pollen
ARG 39	JX905506	ARG, Corrientes Prov., Chañar Stream	aquatic	pollen
ARG 97	JX905513	ARG, Tierra del Fuego, Rancho Grande Peat Bog	peat bog	pollen
ARG 122	JX905520	ARG, Entre Rios Prov., Departamento de Villaguay, roadside pond	aquatic	pollen

BR 97	AY439074	Ramsayville Marsh, near Ottawa CANADA)	aquatic	moribund algae
JA 3	KC691314	USA, AL, Tuscaloosa, Lake Nicol	aquatic	pollen
JA 8	KU721090	USA, AL, Hale County, Vernal Pool, TNF[1]	soil	keratin
JEL 176	AY439064	USA, ME, Augusta, Viles Arboretum	aquatic	keratin
MP 5	AY988511	USA, MI, Pellston, Douglas Lake,	aquatic	pollen
		Michigan Biological Station		
MP 41	JX905522	USA, AL, Tuscaloosa, West Side Stream,	aquatic	pollen
		Myriophyllum roots		
MP 59	JX905525	USA, AL, Wheeler Wildlife Refuge, pond	aquatic	chitin
MP 60	*MK543212	USA, AL, Wheeler Wildlife Refuge, pond	aquatic	chitin
MP 61	*MK543213	USA, AL, Wheeler Wildlife Refuge, pond	aquatic	chitin
MP 66	KC691342	USA, AL, Northport, Road side ditch	aquatic	pollen
		with *Spirogyra*		
MP 69	JX905526	USA, AL, Old Northport, Trestle, soil	moist soil	pollen
		with *Vaucheria*		
MP 70	KC691345	USA, AL, Cottondale, Rainey Pond	aquatic	pollen
MP 72	*MK543214	USA, AL, Wagarville, Crawfish Pond	aquatic	chitin
MP 80	KC691348	USA, AL, Cottondale	moist soil	pollen
MP 89	KC691354	USA, AL, Tuscaloosa, Black Warrior River	aquatic	pollen
PL AUS 5	AY988512	AUS, NSW, Wingecarribe Shire,	stream	pollen
		Fitzroy Falls		
PL AUS 14	AY422956	AUS, NSW, Ourimbah	soil	pollen
PL AUS 23	AY988514	TASMANIA, Sarah Island	soil	pollen
PL 148	*MK543218	USA, TX, Livingston, Alabama-Coushatta	roadside	pollen
		Indian Reservation	ditch	
PL 149	KF257912	USA, TX, Cold Spring, Sam Houston	aquatic	pollen
		National Forest, Double Lake		
WB 216	KC691358	USA, AL, Cottondale, Rainey Pond	aquatic	moribund algae
WB 235A	DQ536493	USA, AL, Coker, Lake Lurleen,	aquatic	dragonfly wing
		under alder tree		
WB 241	KC691366	USA, AL, Northport, North Wood Lake,	mud	chitin
		Union Chapel Rd., *Myriophyllum roots*		
WB 266D	*MK543215	USA, NC, Lake Lure, Broad River, Bill Mt.	aquatic	chitin
WB 266E	*MK543216	USA, NC, Lake Lure, Broad River, Bill Mt.	aquatic	chitin
WB 266F	*MK543217	USA, NC, Lake Lure, Broad River, Bill Mt.	aquatic	chitin
WJD 131	JX905530	USA, AL, Cheaha	soil	cellulose
WJD 138	KC461388	USA, AL, Tuscaloosa, Black Warrior River	aquatic	pollen

WJD 183	KU721100	USA, AL, Hale County, Vernal Pool, TNF [1]	aquatic	dragonfly wing
JEL 794	*MK558057	USA, ME, Mud Pond, Hancock County	aquatic	chitin
		Outgroup		
JEL 222	DQ485551	*Rhizophydium globosum* USA, ME, Orono	soil	pollen
[1]Oakmulgee District of the Talladega National Forest (TNF) *Newly generated sequences				

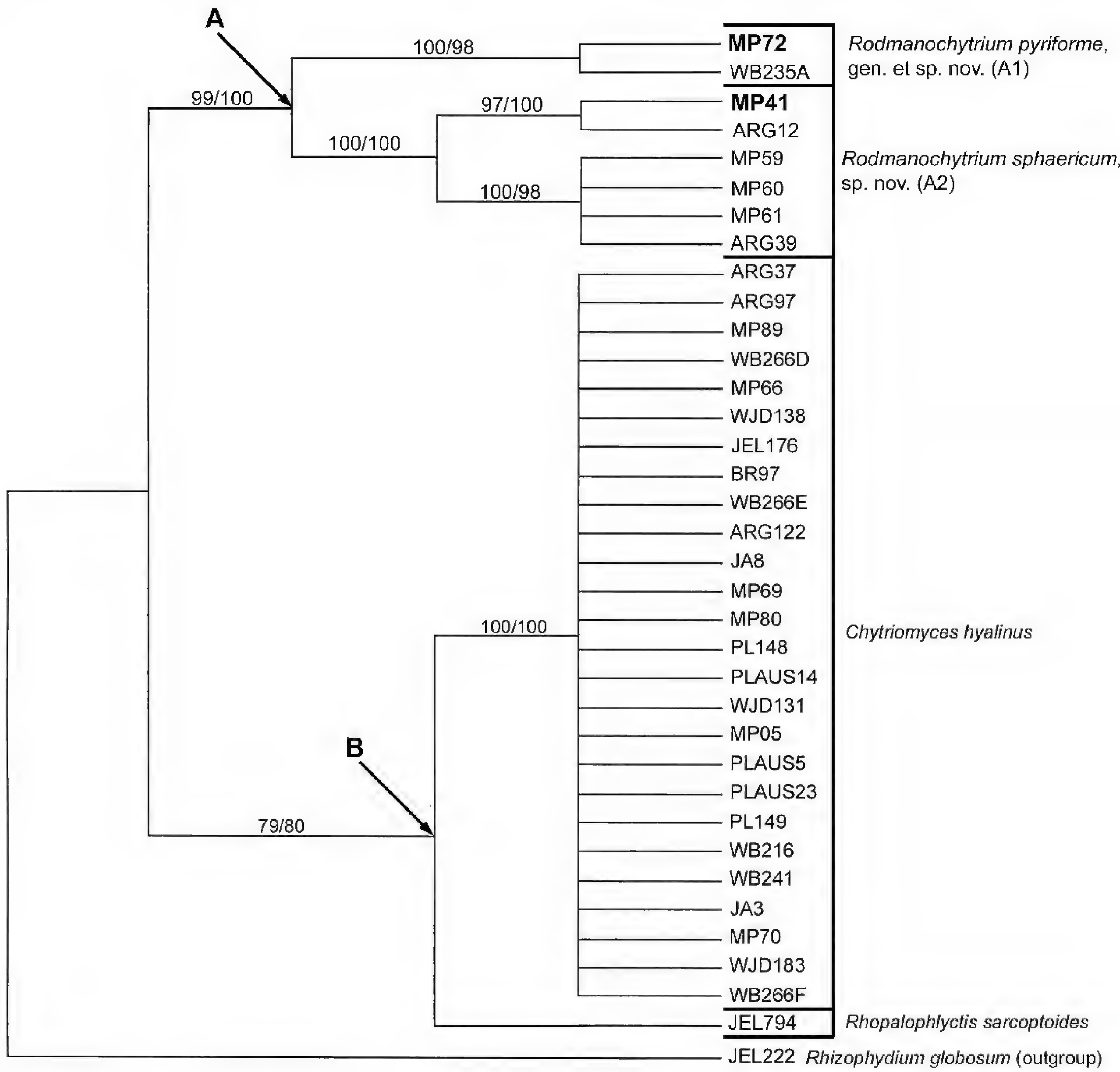

Figure 1. Molecular phylogenetic assessment. Cladogram inferred from strict consensus, maximum parsimony analysis of 35 strains in *Chytriomycetaceae* using 28S rDNA sequences. Numbers at nodes are bootstrap support values (maximum likelihood/maximum parsimony). Lineage A includes eight unidentified chitinophilic strains in two sub-clades (A1, A2), which represent *Rodmanochytrium pyriforme* gen. et sp. nov. (A1) and *R. sphaericum* sp. nov. (A2). Lineage A is sister of Lineage B. Lineage B includes representatives of two other chitinophilic species, *Rhopalophlyctis sarcoptoides* and the morpho-species *Chytriomyces hyalinus*. Strain JEL 222 *Rhizophydium globosum* is the outgroup.

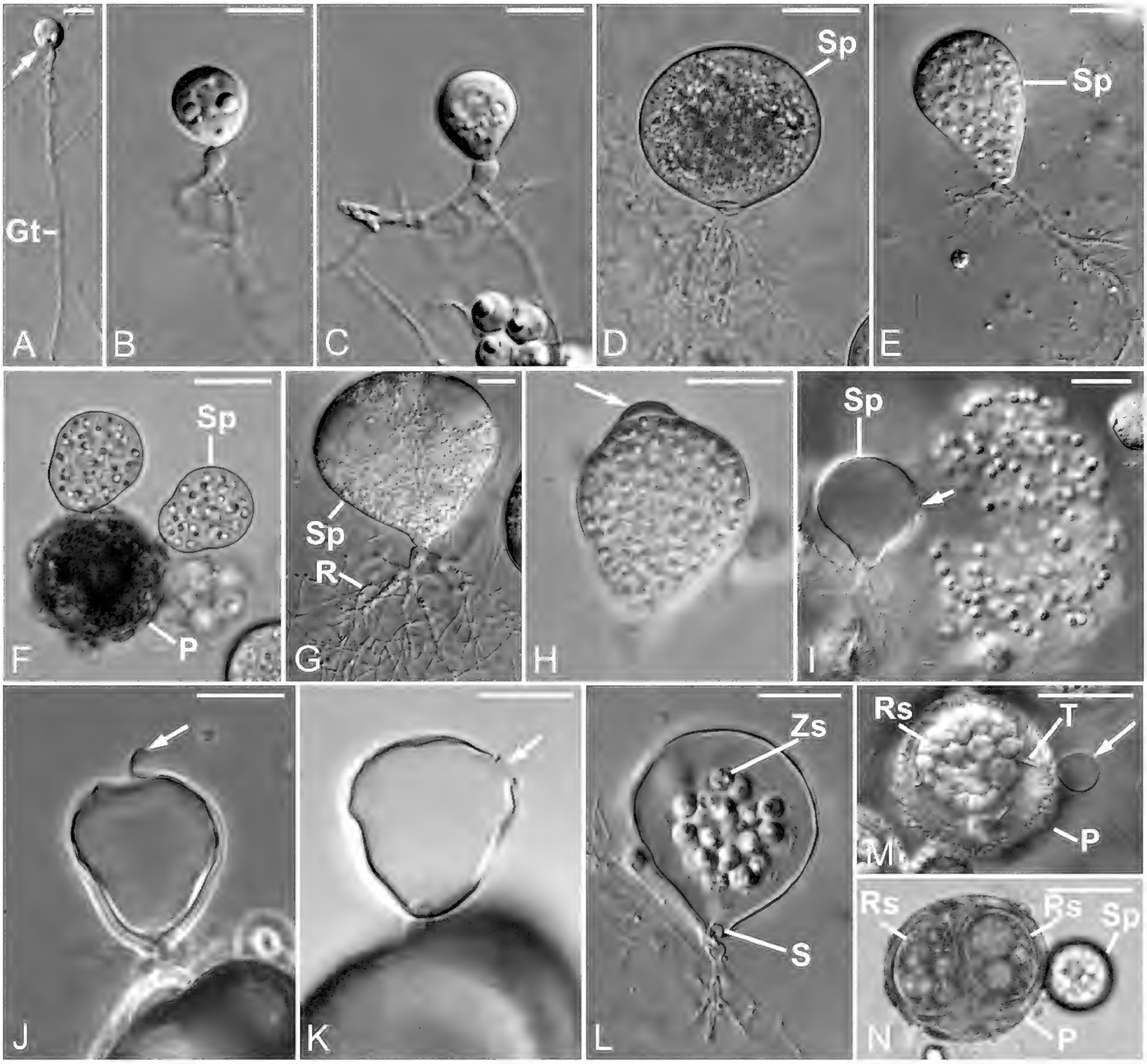

Figure 2. Light microscopy of thallus morphology of *R. pyriforme* sp. nov., strain MP 72. (A) Encysted zoospore (arrow) with long germ tube. (B) Germling with developing rhizoid; sub-sporangial portion of rhizoid spherical, dichotomously branching near enlarging encysted zoospore. (C) Developing thallus; incipient sporangium pyriform; sub-sporangial portion of rhizoidal axis broadly tubular; rhizoids branch dichotomously. (D) Oval sporangium with apical portion broadest and tapering toward the base. Rhizoids finely branched. (E) Clavate sporangium tapering toward base and containing cleaved zoospores. (F) Pollen grain with two pyriform sporangia attached. (G) Pyriform sporangium with narrow base and finely branched rhizoids. (H) Gelatinous plug formed below the forming operculum (arrow). (I) Vesicular zoospore discharge; empty sporangium is pyriform with lateral discharge pore (arrow). (J) Empty pyriform sporangium on pollen with operculum still attached to side of discharge pore (arrow). (K) Empty pyriform sporangium on pollen grain; sub-apical discharge pore with recurved edge (arrow). (L) Partially empty pyriform sporangium with dome-shaped septum. (M) Spherical resting spore in pollen; empty zoospore cyst at surface of pollen grain (arrow) with tube connected to resting spore. (N) Two oval resting spores inside of a pollen grain; developing sporangium attached to the surface of the pollen grain. Scale bar = 5 μm (A), 10 μm (B, C, G), 20 μm (D, E, F, H-N). Abbreviations: Gt, germ tube; P, pollen; R, rhizoid; Rs, resting spore; S, septum; Sp, sporangium; T, tube; Zs, zoospore.

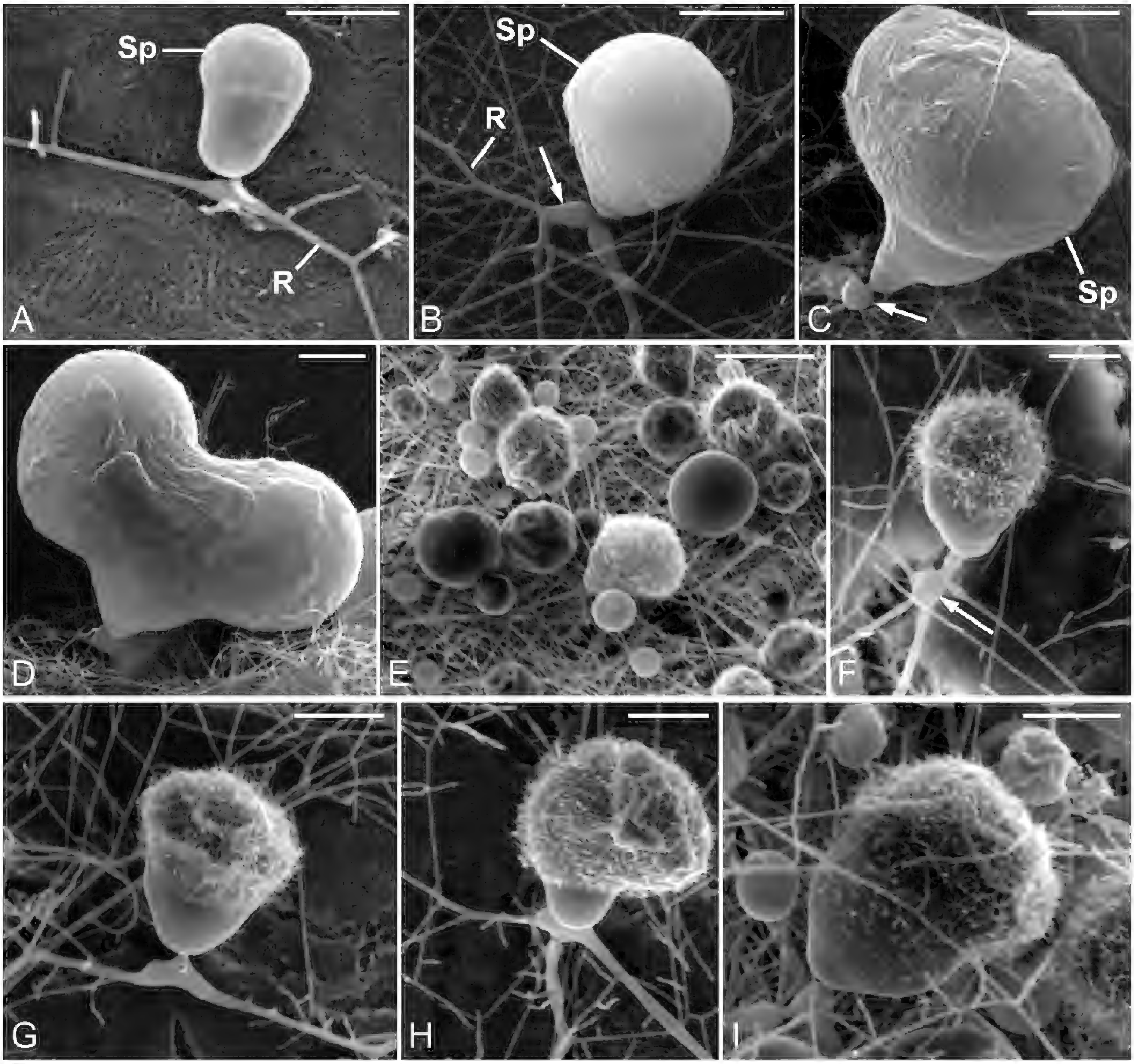

Figure 3. Scanning microscopy of thallus morphology of *R. pyriforme* sp. nov., strain MP 72; chitin substrate. (A, B) Developing pyriform sporangia, surfaces smooth. (C) Pyriform sporangium with rugose upper surface and spherical sub-sporangial rhizoidal connection (arrow). (D) Lobed sporangium with rugose surface. (E) Sporangia with smooth and hirsute surfaces. (F) Developing thallus; upper surface hirsute; rhizoid arises on sporangium as a short tube from a single point and with a small swelling formed (arrow). (G, H, I) Developing hirsute thalli; upper portion of sporangia covered with fine hair-like structures. Rhizoids branch near sporangium. Scale bar = 5 µm (A, F-I), 10 µm (B, C, D), 30 µm (E). Abbreviations: R, rhizoid; Sp, sporangium.

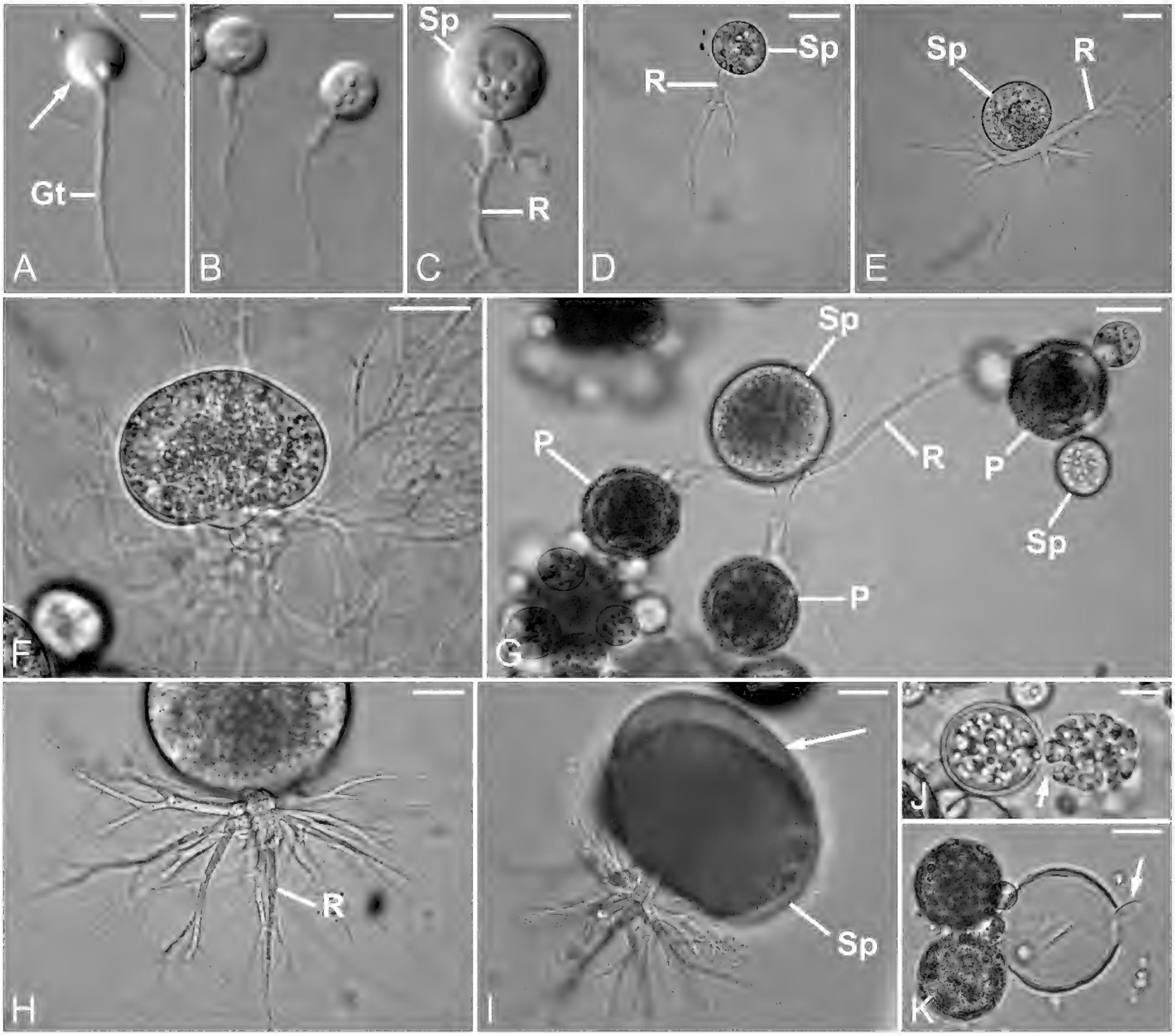

Figure 4. Light microscopy of thallus morphology of *R. sphaericum* sp. nov., strain MP 41. (A) Encysted zoospore (arrow) with elongate germ tube. (B) Two germlings; rhizoidal axis swollen at base of incipient sporangium. (C) Developing thallus; incipient sporangium spherical; rhizoidal axis swollen near sporangium and dichotomously branched. (D) Developing thallus; trunk-like main rhizoidal axis extends with fine branches. (E) Developing thallus; rhizoidal axis stout near sporangium and branching dichotomously. (F) Oval sporangium; basal portion cup-shaped; sub-sporangial rhizoidal axis connection bulbous. (G) Thallus development on pollen illustrating range in sporangial diameters; small epibiotic sporangia; larger interbiotic thallus; sporangia spherical. (H) Rhizoidal system arises from short sub-sporangial tube as stout branches; branching dichotomously into fine branches. (I) Oval sporangium with bushy rhizoidal system. Cytoplasm in sporangium forms zones (arrow). (J) Vesicular zoospore discharge (arrow) from spherical sporangium. (K) Empty spherical sporangium with operculum attached to rim of discharge pore (arrow). Scale bar = 5 µm (A), 10 µm (B, C, J, K), 20 µm (D, E-I). Abbreviations: Gt, germ tube; P, pollen; R, rhizoid; Sp, sporangium.

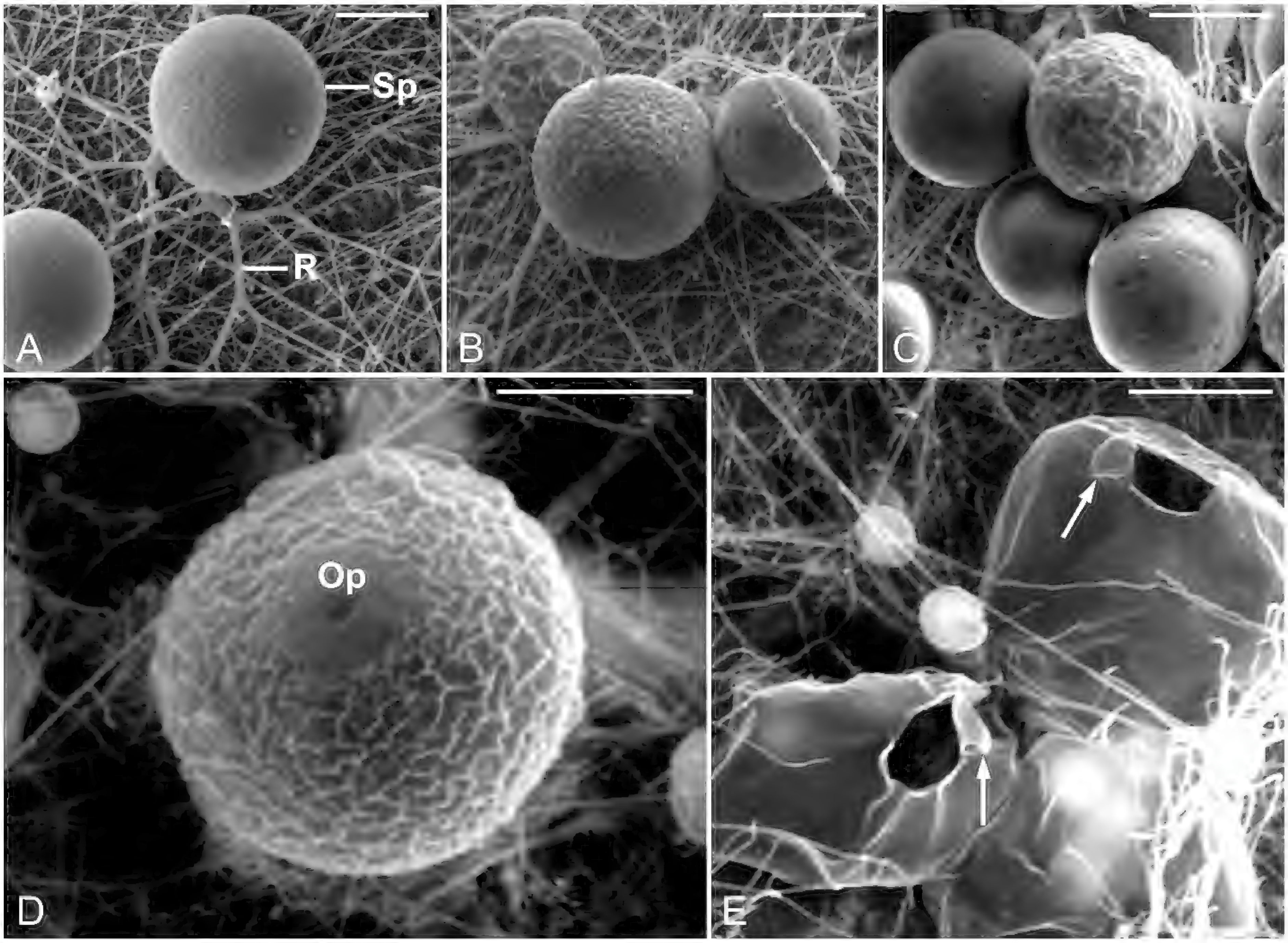

Figure 5. Scanning electron microscopy of thallus morphology of *R. sphaericum* sp. nov., strain MP 41; chitin substrate. (A) Spherical sporangium with finely-granular surface; rhizoidal axis near sporangium stout and branching dichotomously. (B) Sporangia with finely-granular, reticulate and smooth surfaces. (C) Sporangia with reticulate and smooth surfaces. (D) Sporangium with reticulate surface; area over the operculum is smooth. (E) Empty sporangia with smooth surfaces; operculum still attached to the side of the discharge pore (arrow).
Scale bar = 10µm (A-E). Abbreviations: Op, operculum; R, rhizoid; Sp, sporangium.

Essential oils of whole tree, trunk, limbs and leaves of *Juniperus osteosperma* from Utah

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ABSTRACT

Volatile oil produced through steam distillation of *Juniperus osteosperma* was examined to establish essential oil yields and aromatic profiles from the whole juniper tree as well as each portion of the tree, namely the trunk, limbs, and leaves. The whole tree aromatic profile is abundant in α-pinene (40.5%), sabinene (4.7%), ρ-cymene (2.7%), limonene (4.2%), camphor (6.7%), bornyl acetate (6.7%), cis-thujopsene (5.6%), and cedrol (2.9%). Trunk essential oil is prominent in α-pinene (59.4%), δ-3-carene (4.4%), cis-thujopsene (11.0%) and cedrol (3.0%). Limb essential oil is prominent in α-pinene (85.3%) and cis-thujopsene (1.3%). Leaf essential oil, in agreement with previous findings, was composed primarily of lighter fractions, prominent in camphor (28.3%) and bornyl acetate (15.8%). The greatest yield (w/w) is from the leaf material (0.8%), followed by the whole tree (0.3%). The trunk (0.1%) and limbs (0.1%) provide the lowest yields. Published on-line www.phytologia.org *Phytologia 101(3): 188-193 (Sept 21, 2019)*. ISSN 030319430.

KEY WORDS: *Juniperus osteosperma*, aromatic profile, essential oil, trunk, limbs, leaves, yield.

The genus *Juniperus* consists of nearly 75 species (Adams and Schwarzbach, 2013, Adams 2014). *Juniperus osteosperma* (Torr.) Little is a one-seeded, serrate leaf margined juniper (Adams et al., 2006) that has a widespread distribution throughout Utah, found in all counties in the state (Welsh, 1993).

The essential oil profile for *J. osteosperma* heartwood was previously established as being prominent in cis-thujopsene and cedrol (Adams, 1987) and leaf oil being prominent in camphor and bornyl acetate (Adams, 2012), with a significant degree of observed profile variation in the leaf oil. The latter study determined that differences in leaf essential oil profile were likely associated with geographic location. Commercially available whole tree *J. osteosperma* essential oil from Young Living Essential Oils, the sole producer, is produced from chipped plant material consisting of trunk, limb, and leaf parts distilled together.

The present study establishes the aromatic profile for whole tree, trunk, and limb essential oils and confirms previously established aromatic profiles for leaf essential oils of *J. osteosperma*. Essential oil yields for the whole tree and each portion of the tree are also examined.

MATERIALS AND METHODS

Juniperus osteosperma plant material was collected from ten locations, one tree per site, throughout the state of Utah. Samples collected were from trees with approximate dimensions of 4 m height, 4 m width (foliage), and 0.25 m diameter (trunk). For leaf material, approximately 100 g total was cut from four sides of the tree, approximately 2 m above the ground. A single whole tree was cut down in order to obtain samples of trunk, limbs, and leaves. The trunk is defined as a 0.25 m section (0.25-0.5 m above ground) including heartwood, sapwood, cambium, and bark. The limb is defined as leafless, 3-5 cm diameter sections nearest the trunk, approximately 2 m above ground. Voucher samples are held in the

Utah Valley University Herbarium (UVSC): *J. osteosperma* (Torr.) Little, Wilson 2018-01, -02, -03, -04, -05, -06, -07, -08, -09, -10 (UVSC).

Samples of whole tree (n=1), trunk (n=1), limb (n=1), and leaf (n=10) of *J. osteosperma* were processed as follows for laboratory scale distillation: each mentioned portion of the tree was cut, chipped, stored at ambient temperature out of direct sunlight, and steam distilled within 24 hours.

Laboratory scale distillation was as follows: 3 L of water added to the bottom of a 12 L distillation chamber (Albrigi Luigi S.R.L., Italy), plant material accurately weighed and added to the distillation chamber, distillation for 4 hours by direct steam, essential oil separated by a cooled condenser and Florentine flask. Essential oil samples were filtered and stored in a sealed amber glass bottle until analysis.

Essential oils were analyzed, and volatile compounds identified, by GC/MS using an Agilent 7890B GC/5977B MSD and J&W DB-5, 0.25 mm x 60 m, 0.25 µm film thickness, fused silica capillary column. Operating conditions: 0.1 µL of neat sample, 150:1 split ratio, initial oven temperature of 40 °C with an initial hold time of 5 minutes, oven ramp rate of 4.5 °C per minute to 310 °C with a hold time of 5 minutes. Volatile compounds were identified using the Adams volatile oil library (Adams, 2007) using Chemstation library search in conjunction with retention indices. Note that limonene/β-phellandrene and myrtenol/myrtenal elute as single peaks, but their amounts are determined by the ratio of masses 68 and 79 (limonene), 77 and 93 (β-phellandrene), 108 and 152 (myrtenol), 107 and 150 (myrtenal). Volatile compounds were quantified by GC/FID using an Agilent 7890B and J&W DB-5, 0.25 mm x 60 m, 0.25 µm film thickness, fused silica capillary column. Operating conditions: 0.1 µL of sample (20% soln. for essential oils, 1% for reference compounds), splitless injection with purge flow to split vent 10 mL/min at 0.25 min, initial oven temperature at 40 °C with an initial hold time of 2 minutes, oven ramp rate of 3.0 °C per minute to 250 °C with a hold time of 3 minutes. For quantification, compounds were identified using retention indices coupled with retention time data of reference compounds.

The percent yield was calculated as the ratio of mass of processed plant material immediately before distillation to the mass of essential oil produced.

RESULTS AND DISCUSSION

Prominent compounds from steam distilled *J. osteosperma* whole tree essential oil are α-pinene (40.5%), sabinene (4.7%), ρ-cymene (2.7%), limonene (4.2%), camphor (6.7%), bornyl acetate (6.7%), cis-thujopsene (5.6%), and cedrol (2.9%) (Table 1). All volatile compounds present in the essential oils of *J. osteosperma* trunk, limb, and leaf, separately distilled, are also present in the essential oil distilled from the whole tree, albeit at different relative area percentages. Prominent compounds from steam distilled trunk essential oil include α-pinene (59.4%), δ-3-carene (4.4%), cis-thujopsene (11.0%), and cedrol (3.0%). Prominent compounds from steam distilled limb essential oil include α-pinene (85.3%) and cis-thujopsene (1.3%). Camphor (28.3%) and bornyl acetate (15.8%) are the prominent compounds detected in the leaf essential oil (Table 1).

In *J. osteosperma* trunk essential oil, cis-thujopsene is present at 11.0%. In contrast, cis-thujopsene in leaf essential oil samples is either not detected or in trace amounts. When comparing different portions of the tree, the relative amount of cis-thujopsene detected is most abundant in samples near the heartwood (Table 1). From trunk to limb to leaf, the relative amount of cis-thujopsene decreases from 11.0%, to 1.3%, to trace and the relative amount of cedrol decreases from 3.0% to 0.4% to undetected, respectively.

The whole tree, trunk, limbs, and leaves have the following essential oil yields (w/w) respectively: 0.3%, 0.1%, 0.1%, and 0.8%. Each portion of a single whole tree was separated and weighed in order to calculate the distribution of mass and essential oil yield of the trunk, limb, and leaf material (Table 2).

Leaf samples (n=10) distilled throughout the eight-month period showed stark variation for camphor (10.9% to 47.6%) and bornyl acetate (3.0% to 27.7%) (Figure 1). These results and the variability observed in samples throughout the state of Utah (Figure 2) agree with previous findings of *J. osteosperma* leaf essential oils (Adams, 2012).

CONCLUSIONS

The whole tree *J. osteosperma* essential oil profile is abundant in α-pinene, sabinene, ρ-cymene, limonene, camphor, bornyl acetate, cis-thujopsene, and cedrol. Trunk essential oil is prominent in α-pinene, δ-3-carene, cis-thujopsene, and cedrol. Limb essential oil is prominent in α-pinene and cis-thujopsene. The leaf material provides the greatest yield (w/w), follow by the whole tree. The trunk and limbs provide the lowest yields.

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Table 1. Aromatic profile of *J. osteosperma* essential oil from the whole tree, trunk, limbs, and leaves (n=1 for each sample type). Compounds detected in the whole tree but not in select portions of the tree are denoted as not detected (nd) and values less than 0.1% as traces (t). Unidentified compounds less than 0.5% are not included. KI is the Kovat's Index using a linear calculation on DB-5 column. Relative area percent as determined by GC/FID.

KI	Compound	Whole Tree	Trunk	Limb	Leaf
921	tricyclene	1.9	0.2	0.4	2.6
924	α-thujene	0.7	t	t	0.6
932	α-pinene	40.5	59.4	85.3	3.7
945	α-fenchene	0.1	0.6	0.3	t
946	camphene	1.5	0.3	0.4	2.5
953	thuja-2,4(10)-diene	0.5	0.4	0.5	t
969	sabinene	4.7	0.3	0.5	8.8
974	β-pinene	0.5	0.6	0.8	0.2
988	myrcene	1.3	1.0	1.0	2.1
1002	α-phellandrene	0.3	t	nd	0.1
1008	δ-3-carene	0.5	4.4	1.1	nd
1014	α-terpinene	0.8	t	t	0.9
1020	ρ-cymene	2.7	0.3	0.3	2.3
1024	limonene	4.2	0.5	0.9	10.6
1025	β-phellandrene	0.7	0.3	0.1	0.4
1044	trans-β-ocimene	0.3	0.2	0.3	0.2
1054	γ-terpinene	1.3	t	t	1.5
1065	cis-sabinene hydrate	0.1	nd	nd	0.3
1089	ρ-cymenene	0.8	0.5	0.2	0.7
1098	trans-sabinene hydrate	0.1	nd	nd	1.0
1122	α-campholenal	0.3	0.2	0.3	t
1135	trans-pinocarveol	0.6	0.5	0.6	0.4
1141	camphor	6.7	0.3	0.3	28.3
1145	camphene hydrate	0.2	nd	nd	1.7
1160	pinocarvone	0.1	0.2	0.2	nd
1165	borneol	1.6	t	t	3.9
1174	terpinen-4-ol	2.0	t	t	4.7
1186	α-terpineol	0.3	1.1	t	0.2
1194	myrtenol	0.2	0.2	0.1	t
1195	myrtenal	0.2	0.5	0.3	t
1204	verbenone	0.3	0.3	0.3	0.1
1215	trans-carveol	0.2	nd	0.1	0.5
1239	carvone	0.2	nd	t	0.5
1241	carvacrol, methyl ether	0.3	1.3	0.7	nd
1283	isobornyl acetate	0.4	nd	nd	0.7

KI	Compound	Whole Tree	Trunk	Limb	Leaf
1284	bornyl acetate	6.7	0.3	0.9	15.8
1410	α-cedrene	1.3	0.8	0.1	nd
1417	trans-caryophyllene	0.2	0.1	0.1	nd
1419	β-cedrene	0.2	0.5	t	nd
1429	cis-thujopsene	5.6	11.0	1.3	t
1452	α-humulene	0.1	nd	t	nd
1465	thujopsadiene	0.2	0.2	nd	nd
1498	pseudowiddrene	0.1	0.2	nd	nd
1504	cuparene	0.2	0.3	t	nd
1513	γ-cadinene	0.3	0.6	nd	nd
1522	δ-cadinene	0.4	0.2	nd	0.1
1548	elemol	0.8	t	0.1	1.9
1582	caryophyllene oxide	0.2	nd	0.1	nd
1589	allo-cedrol	0.1	0.2	nd	nd
1599	widdrol	0.5	1.7	0.1	nd
1600	cedrol	2.9	3.0	0.4	nd
1630	γ-eudesmol	0.3	0.7	0.2	0.2
1649	β-eudesmol	0.3	0.7	0.3	0.4
1652	α-eudesmol	0.1	0.4	0.2	t
1688	cedr-8-en-13-ol	0.2	0.4	nd	nd

Table 2. Distribution of mass and essential oil (EO) yield from a single *J. osteosperma* tree (the number was limited to one as per permit restrictions). Tree was cut 0.25 m above ground; all measurements and calculations are reflective of above ground portions.

	Whole tree	Trunk	Limb	Leaf
mass (kg)	209.5	77.9	68.1	63.5
mass (%)	100.0	37.2	32.5	30.3
mass distilled (g)	1141.3	1929.6	1741.3	1200.4
yield EO (g)	3.5	2.3	1.7	10.1
yield EO (%)	0.3	0.1	0.1	0.8

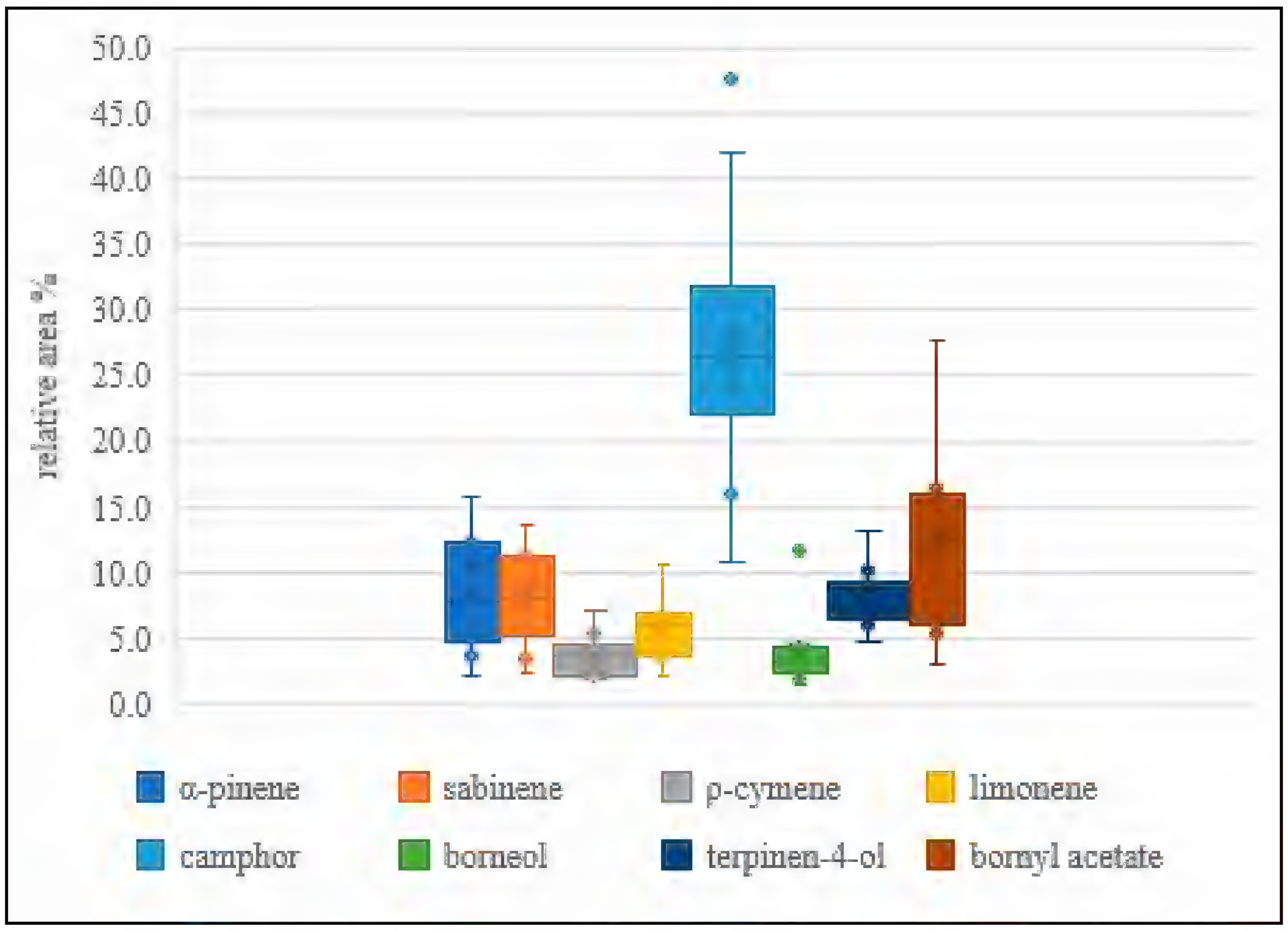

Figure 1. Average aromatic profile for *J. osteosperma* leaf essential oil (n=10) over an 8-month period. Compounds shown are abundant in the essential oil. Extreme outliers were present for camphor (mean of 27.4, outlier of 47.6) and borneol (mean of 4.0, outlier of 11.8). Relative area percent as determined by GC/FID.

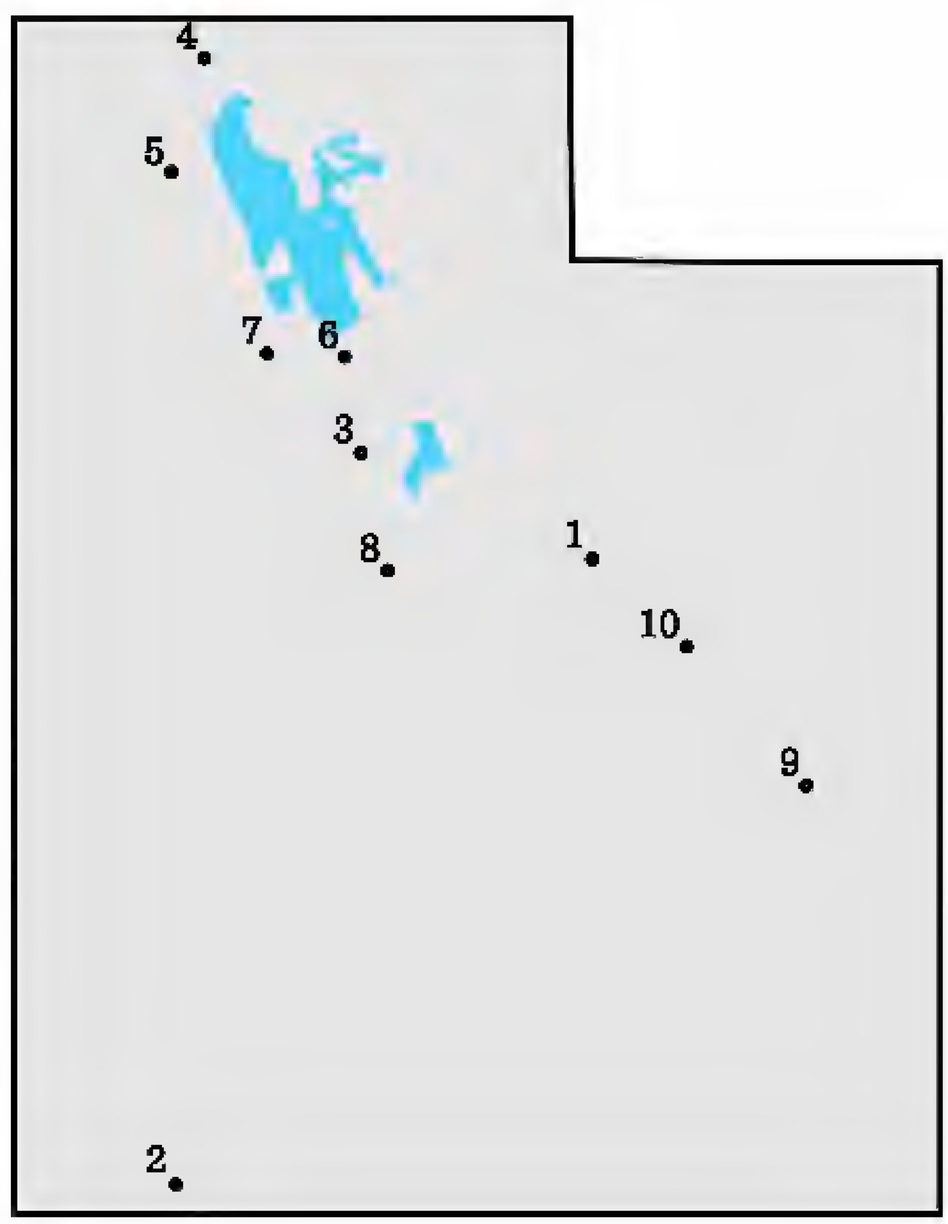

Figure 2. Map showing collection sites of *J. osteosperma* plant material. Samples were collected in sequence, 1-10. The following are sample collection dates and elevation: 1) 27 Mar 2018, 2140 m; 2) 8 May 2018, 1548 m; 3) 31 May 2018, 1667 m; 4) 30 Jul 2018, 1427 m; 5) 30 Jul 2018, 1509 m; 6) 30 Jul 2018, 1524 m; 7) 30 Jul 2018, 1448 m; 8) whole tree, 29 Aug 2018, 1744 m; 9) 5 Oct 2018, 1420 m; 10) 5 Oct 2018, 1631 m.

The effects of plant growth regulator (methyl jasmonate), salt (NaCl) stress and nutrient deficiency on biomass and hydrocarbon yields in *Helianthus annuus* cv. Munchkin (Asteraceae, Sunflowers)

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ABSTRACT

Sunflowers, *H. annuus* cv. Munchkin, were subjected to 5 different treatments (spraying twice with 100 mM methyl jasmonate (MeJA), stress by watering with 30 mM, 120 mM and 240 mM salt (NaCl) water, and nutrient stress, by growing in sand) to determine their effects on leaf biomass, free hydrocarbons (HC) concentrations, and yields of HC/ g biomass. Biomass was significantly larger than the control for plants sprayed twice with methyl jasmonate (100 µM, each) and harvested 7 days later. Increasing stress by watering with increasingly high concentrations of salt, resulted in slower growth and less biomass. Growing the Munchkin plants in sand (very low nutrients) produced stunted plants with very small amounts of biomass, but with a high % HC yields. Salt concentrations of 30 mM and 120 mM decreased the % HC yields, but 240 mM plants had, statistically, the same % HC yields as the control. Total hydrocarbon yield (as g HC/ g DW 10 leaves) was largest in MeJA spraying, followed by the control, with no significant differences among salt concentrations, followed by very small yields under nutrient stress (sand treatment). Published on-line www.phytologia.org *Phytologia 101(3): 194-199 (Sept 21, 2019)*. ISSN 030319430.

KEY WORDS: *Helianthus annuus,* Sunflower, methyl jasmonate, effects on hydrocarbon yields.

Soil salinization is said to affect over 100 countries in the world with varying levels and agricultural responses. High soil salinity levels in farmland can cause plant stress responses so detrimental that these lands are unusable. These unusable lands have an impact on not only agricultural production in these areas, but environmental health and economic welfare as well (Renegasamy 2006). Salt (NaCl) is an abiotic compound that is hydrophilic and as such, attracts water and retains it in the soil resulting in less available water for plants to absorb (Renegasamy, 2006). Plants then go into a drought stress-response phase which may change the metabolites. Responses include a myriad of mechanisms causing stunted growth, decreased dry weight, and a reduced number of leaves (Khalid and Ahmed 2017).

Salt stress has been found to increase secondary metabolite production in various plants such as *Stevia rebaudiana* Bertoni*, Apocyni veneti* Folium, and *Nigella sativa* L (Chen et al. 2013; Khalid 2017; Chen et al. 2018).

Studies on the effects of salt solutions as the water source for growing *Chrysanthemum* X*morifolium* showed stunted plants in all NaCl test groups and the reduction of dry shoot weight in some groups (van Iesrel and Lee 2008).

Salt tolerance was tested on coastal and inland *Grindelia camporum* and *G. stricta (Asteraceae*) using concentrations ranging from 5mM to 550 mM, with the highest being near that of seawater (600 mM). Plants subjected to higher salt concentrations had significantly decreased biomass (Ravetta, D., et. al., 1997). Interestingly, crude resin content (%) declined in the 125 mM treatment, but increased in the 250 mM treatment after 84 days (Ravetta et. al. 1997). Plants recognize stress at a cellular level and

may respond by producing different amounts of secondary metabolites. such as hydrocarbons, phenyl amides, and polyamines (Ramakrishna and Ravishankar 2011).

In a paper on the induction of sesquiterpene lactone (STL) defenses in *Helianthus annuus*, by methyl jasmonate (MeJA), Rowe, Ro and Rieseberg (2012) reported MeJA treated sunflower plants had a lower STL production and lower glandular trichome density. This is in contrast to other studies that have found MeJA to induce increased concentrations of terpenoids in cotton (*Gossypium hirsutum*, Opitz, Kunert and Gershenzon, 2008), *Tanacetum parthenium* (Majdi et al. 2015) and see review on the roles of MeJA in plants by Browse (2005).

Defense chemicals are both constitutive and inducible defenses (see Wittstock and Gershenzon, 2002 for discussion). Recently, we reported (Adams et al. 2017c) that progeny of high hydrocarbon (HC) yielding sunflower (*H. annuus*) populations displayed much reduced HC yields when grown in greenhouse conditions. We reported the percent HC (greenhouse / field grown HC yields) decreased to 45.9, 55.6 and 78.3%. In addition, g HC / g DW weights of leaves were very reduced to from 17.9 g tp 6.1 g when plants were grown in a greenhouse. It appears that biotic and abiotic factors in natural populations can have large effects on HC yields.

The purposes of the present paper are to report the effects of plant growth regulator (methyl jasmonate, MeJA), salt (NaCl) stress and nutrient deficiency on biomass, % HC yields and HC yields as gHC/ g DW leaves.

This report is a part of a continuing study on the development of sunflowers as a source for natural rubber and bio-fuels from the biomass (Adams et al., 1986; Adams and Seiler, 1984; Adams and TeBeest, 2016; Adams et al. 2016; Adams and TeBeest, 2017; Adams et al. 2017a,b,c; Adams et al. 2018a,b,c; Pearson et al., 2010a,b; Seiler, Carr and Bagby, 1991,).

MATERIALS AND METHODS

Seeds of *H. annuus* cv. Munchkin were obtained from Sunflower Selections, Inc., Woodland, CA. Seeds were planted in 6 " square plastic pots using Miracle Grow® potting soil. Plants were grown in a growth chamber with LED lighting approximately equal to daylight for 16 hr light, 8 hr dark cycles and watered as needed.

Ten plants were used for each treatment (Table 1). Munchkin seeds were planted on Jan. 7 in soil saturated with tap water. All plants were watered with 200 ml tap water on Jan. 17, and 21, then with 300 ml tap water on Jan. 25, 29, and Feb. 1. Control plants were watered with 300 ml tap water, and salt (NaCl) treatments were watered with 300 ml of 30 mM, 120 mM and 240 mM of salt solutions on Feb. 4, 7, 12, 14. On Feb. 18 all plants (control, salt treatment) were watered with 300 ml of tap water and 10 leaves were harvested from each plant on Feb. 21.

Concurrent with the salt concentration experiment, 10 plants were grown in a low-nutrient soil mixture consisting of sand/ potting soil (7:1). Munchkin seeds were planted on Jan. 7 in soil saturated with tap water. Due to the fine texture of the sand and slow growth, the plants were not watered on Jan. 17, or Jan. 21. Plants were watered with 200 ml of tap water on Jan. 25, 29, Feb. 1, 4, 7, 12, 14, and 18. Plant leaves (10) were harvested on Feb. 21 (4 days after last watering).

In addition, 10 plants were grown with the aforementioned control and watered exactly as the control. On Feb. 22 these 10 plants were sprayed with 100 µM methyl jasmonate with a hand sprayer until each leaf was wet then let dry, then sprayed again. Leaves were harvested on March 1, 7 days after spraying.

The 10 lowest growing, non-yellowed, mature leaves were collected. The leaves were air dried in paper bags at 49° C in a plant dryer for 24 hr or until 7% moisture was attained.

Leaves were ground in a coffee mill (1mm). 3 g of air-dried material (7% moisture) were placed in a 125 ml, screw cap jar with 20 ml hexane, the jar was sealed, then placed on an orbital shaker for 18 hr. The hexane soluble extract was filtered through a Whatman paper filter into a pre-weighed aluminum pan and the hexane evaporated on a hot plate (50°C) in a hood. The pre-weighed aluminum pan with concentrated hydrocarbon extract was weighed and tared. Extraction of identical samples by shaking and soxhlet (8 hr) yielded a correction factor of 1.9 (soxhlet yield/ shaking yield), which when corrected to oven dry weight basis (ODW) by 1.085 resulted in a total correction factor of 2.06.

ANOVA and SNK (Student Newman-Keuls) multiple range tests were programmed following the formulations in Steel and Torrie (1960).

RESULTS

Table 1 shows the results from the treatments ANOVA and SNK statistical analyses. Biomass was highly significantly higher for methyl jasmonate (MJAS) sprayed plants. This is in contrast to a previous study (Adams and Johnson 2019) that reported biomass in Munchkin was not affected by the application of a single spraying of 100 µM MeJA, and harvested after 2 days, and 4 days. However, after 14 days, biomass was significantly larger, as the plants recovered from MeJA treatment and began to grow. It appears that the application of 2 sprays of 100 µM methyl jasmonate seems to initiate growth.

Table 1. Comparison of dry weight (10 leaves), percent HC yields, and g HC/ g DW 10 leaves for cv. Munchkin, subjected to 5 treatments and analyzed after 4 days. Mean values with the same suffix letter are not significantly different (P= 0.05).

	MJ	CT	salt 30 mM	salt 120 mM	salt 240 mM	sand	F ratio significance
Biomass, g DW 10 leaves	3.47a	2.83b	2.66bc	2.54c	2.28d	0.77e	F= 126.9 $P = 0.12^{-9}$ ***
	sand	MJ	CT	salt 240 mM	salt 30 mM	salt 120 mM	F ratio significance
% HC yield	5.96m	5.58m	5.53m	5.25m	4.63n	4.55n	F= 8.36 $P = 0.38^{-4***}$
	MJ	CT	salt 30 mM	salt 240 mM	salt 120 mM	sand	F ratio significance
g HC/ g DW 10 leaves	0.193w	0.155x	0.123y	0.119y	0.116y	0.046z	F= 72.60 $P = 0.67^{-9***}$

Increasing stress by watering with increasingly high concentrations of salt (NaCl) resulted in slower growth and less biomass (Table 1). Growing the Munchkin plants in sand (very low nutrients) produced stunted plants with very small amounts of biomass, but with a high % HC yields (Table 1). Salt concentrations of 30 mM, and 120 mM decreased the % HC yields, but 240 mM plants had, statistically, the same % HC yields as the control. This seems due to the low biomass production.

Hydrocarbon yield (as g HC/ g DW 10 leaves, Table 1) is the product of biomass x % HC yield and was largest in MeJA, followed by the control, with no significant differences among salt concentrations, followed by very low biomass in the nutrient stress (sand) treatment.

Graphing the yields by treatment reveals some interesting patterns (Fig. 1). Biomass, % HC yields, and g HC yield all decline from MeJA to control, 30 mM, and 120 mM salt (red ellipses, Fig. 1). However, at 240 mM salt (Fig. 1), the % HC yield increases. A similar trend is seen in the sand treatment (nutrient deficiency) with increased % HC yield and very low biomass (Fig. 1).

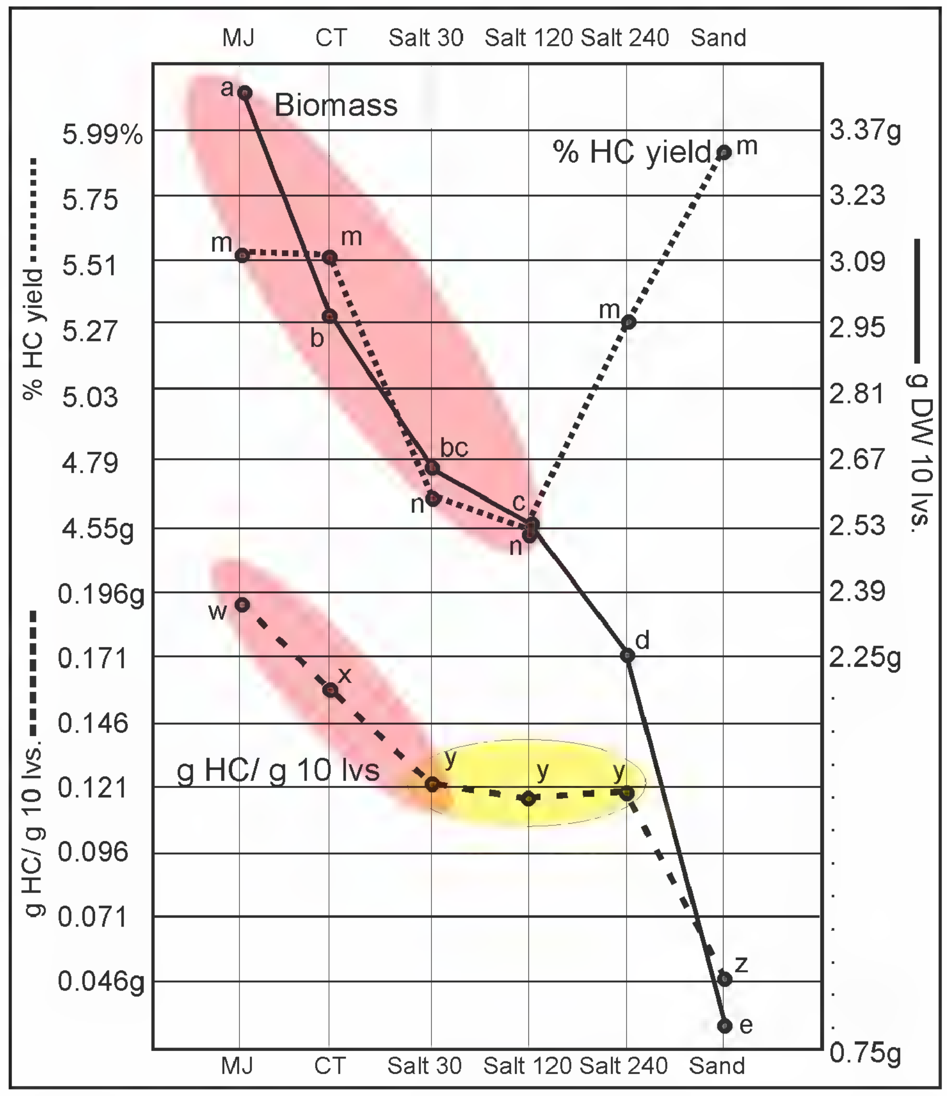

Figure 1. Graphs of dry weight (10 leaves), percent HC yields, and g HC/ g DW 10 leaves for Munchkin subjected to 5 treatments. Means with the same letter superscripts are **not** significantly different (P= 0.05). Similar trends are noted by the red ellipses. The yellow ellipse highlights the uniform HC yields in various salt concentrations. See text for discussion.

It is interesting that total g HC yields are not significantly different among the wide range of salt concentrations utilized in this study (yellow ellipse, Fig. 1). Considering that seawater is approximately 600 mM salinity, the 240 mM salt water is a very high salt concentration (~ 40% of seawater). These results suggest (ignoring environmental effects, etc.) that cultivation using available brackish water, that is common the western US, might be feasible. Although less biomass would be produced, total g HC yields (yellow ellipse, Fig. 1) are not significantly different. Of course, if biomass were used for cellulosic digestion to produce fuel and/ or chemical feedstocks, the use of high amounts of salty water might not be the best economic practice. In addition, irrigation with saline water on the arid lands in the southwest US would hasten the accumulation of salt, rendering the soil unusable.

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Composition of the leaf volatile terpenoids of *Pinus eldarica* Medw. from Azerbaijan compared with *P. brutia* Ten. leaf essential oil

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ABSTRACT

The volatile leaf oil of *Pinus eldarica Medw.,* Azerbaijan, is dominated by β-pinene (20.7%), α-pinene (11.3%), (E)-caryophyllene (13.3%) and germacrene D (9.2%) with moderate amounts of δ-3-carene (2.5%), limonene (3.4), β-phellandrene (2.2), (E)-β-ocimene (3.2), α-terpinyl acetate (2.6), α-humulene (2.4), phenyl-ethyl-3-methyl-butanoate (2.6) and (E)-γ-bisabolene (2.1). Cultivated *P. eldarica* trees (USA, Utah) had a similar profile: germacrene D (27.4%), α-pinene (14.5%), β-pinene (13.4%) and (E)-caryophyllene (10.5%) and with moderate amounts of δ-3-carene (4.5%), limonene (0.9), β-phellandrene (1.3), (E)-β-ocimene (1.6), α-terpinyl acetate (2.3) and α-humulene (2.1). The concentrations of several compounds separate *P. eldarica* and *P. brutia* (α-pinene, camphene, β-pinene, myrcene, (Z)-β-ocimene, α-campholenal, trans-sabinol, camphor, α-terpinyl acetate, (E)-caryophyllene, germacrene D, germacrene D-4-ol, α-cadinene and sandaracopimarinal) and support the practice of recognizing *P. eldarica* (or *P. brutia* ssp. *brutia*) in Azerbaijan and Georgia, where these taxa a part of the native flora. Published on-line www.phytologia.org *Phytologia 101(3): 200-207 (Sept 21, 2019).* ISSN 030319430.
KEY WORDS: *Pinus eldarica,* Azerbaijan, *P. brutia* ssp. *brutia*, volatile leaf oil, terpenes, composition.

The *P. brutia* Ten. complex includes four subspecies: *brutia*, *eldarica*, *pithyusa* and *stankewiczii* (Nahal, 1983; Schiller, 1994; 2000). In the current edition of The Gymnosperm Database (https://www.conifers.org/) Frankis recognizes 5 infraspecific taxa: var. *brutia*, var. *eldarica* (Medw.) Silba, var. *pendulifolia* Frankis, var. *pithyusa* (Steven) Silba, and var. *stankewiczii* (Sukaczev) Frankis. But, in Azerbaijan, *P. brutia* ssp. *eldarica* is recognized at the specific level (*P. eldarica* Medw.). Analysis of cp SSRs in the *halepensis* complex (*P. halepensis* Ait., *P. brutia* and *P. eldarica*) revealed a phylogeny (based on cpSSRs) that placed *P. eldarica* in a significant clade, separate from *P. brutia* (Fig. 2, Bucci et al. 1998), thus, lending some support for the usage of *P. eldarica.* See The Gymnosperm Database (www.conifers.org) and Boydak (2004) for reviews of the taxonomy and nomenclature.

The natural ranges are: ssp. *brutia* - Aegean islands, Crete and Cyprus, and through Turkey to Lebanon and to northern Iraq; ssp. *eldarica* - Caucasus (Azerbaijan, Georgia), and perhaps into northernmost Iran and possibly Afghanistan; ssp. *pithyusa* - in relict stands on the Black Sea coast of the Caucasus mountains; and ssp. *stankewiczii* - Black Sea coast of the Crimea according to Schiller (1994, 2000).

Pinus eldarica has a long history of cultivation dating back to least 500 B.C., when the Persian nobility used it to create forested gardens where few trees could survive. In Persia, the tree was reserved for only the royalty, and became known as the "Tree of Royalty" (Master Gardner Newsletter, July 2010, http://www.darrolshillingburg.com/GardenSite/NewsletterPDF/MG_Newsletters/MGNewsletter_Jul10.pdf). It is commonly grown in the arid southwestern US (s. California, Arizona, s. New Mexico, trans-Pecos Texas and sw Utah) and is sold by the trade names of: Mondell Pine, Mondale Pine, Afgan Pine, Eldarica Pine,

Desert Pine, Goldwater Pine, Elder Pine, and Lone Star Christmas tree. Mondell pine was introduced into the US in 1961 when the USDA obtained five pounds of *P. eldarica* from Afghanistan and gave it to Universities to determine it test its adaptability and potential.

In Azerbaijan it may assume a gnarly shape (Fig. 1) and grows on rock slopes (Fig. 2). In the USA, it is grown as an ornamental tree along streets (Figs. 3, 4) and in gardens. It is said to be short lived in central Texas (10-15 yrs), but in the dry deserts of southwestern US, it is grown under drip irrigation in the arid lands that seem to favor long life, as in Azerbaijan.

Fig. 1. Habit of *Pinus eldarica* in Azerbaijan.

Fig. 2. *P. eldarica* on rock slope, Azerbaijan.

Fig. 3. *P. eldarica* (Mondell Pine) growing, drip irrigated, in Stone Cliff, Utah, USA.

Fig. 4 *P. eldarica* (Mondell Pine) growing as a street tree, drip irrigated, in Stone Cliff, St. George, Utah, USA.

The volatile leaf oils of *Pinus brutia* (usually including *P. eldarica*) have been the subject of several studies (Gohsn et al. 2006; Ioannou et al. 2014; Mitic, et al. 2017; Roussis et al. 1995; Sezik et al. 2008; Vidrich et al. 1999). But, no studies were found on the volatile leaf oil composition of *P. eldarica* from a natural population in Azerbaijan. However, Chubinidze et al. (1999) reported on the monoterpenes from Georgian *P. eldarica* in young vs. old needles and young vs. old cortex (Table 1).

They found the needle oils were dominated α-pinene, δ-3-carene and limonene, and the cortex (wood) oils reflected the same patterns in young and old needles (Table 1).

Table 1. Variation in monoterpene concentrations from leaves and wood cortex of *P. eldarica* from Georgia (former USSR). Adapted from Chubinidze et al. (1999).

cpd\ source	young needles	old needles	young cortex	old cortex
α-pinene	48.8%	78.3	35.8	73.4
camphene	2.1	8.8	2.1	3.2
β-pinene	2.3	5.0	5.4	6.7
δ-3-carene	22.4	3.1	10.9	4.8
limonene	10.2	1.0	20.8	5.6
β-phellandrene	5.0	0.0	25.0	6.0
myrcene	2.1	0.0	0.0	0.0
γ-terpinene	4.1	3.8	0.0	0.0
terpinolene	2.0	0.8	0.0	0.3

Although several other papers cited *P. eldarica* volatile analyses, their samples were obtained from cultivated materials of unknown source. The purpose of this paper is to report on the volatile leaf oil from *P. eldarica* from a natural population in Azerbaijan and compare that oil with other analyses was well as with that of *P. brutia* (ssp. *brutia*) oils.

MATERIALS AND METHODS

Leaf samples collected: *Pinus eldarica*, Azerbaijan, 41° 10' 42.76" N, 46° 13' 55.04" E., 241 m. Coll. *Vahid Farzaliyev 1-9*, 12 April 2019, Lab Acc. *Robert P. Adams 15607-15615(9)*. In addition, samples from cultivated *P. eldarica* (locally called Mondell Pine) were collected from Stone Cliff subdivision, St. George, UT, USA, 22 March 2019, 37° 04' 45" N, 113° 32' 16" W, 844 m, Washington Co., Utah, USA. Coll. *Robert P. Adams 15574-15579(6)*. Voucher specimens are deposited in the herbarium, Baylor University.

Gently dried leaves (100g, 40 - 45˚C) were steam distilled for 2 h using a circulatory Clevenger-type apparatus (Adams, 1991). The oil samples were concentrated (ether trap removed) with nitrogen and the samples stored at -20ºC until analyzed. The extracted leaves were oven dried (100ºC, 48 h) for determination of oil yields.

The oils were analyzed on a HP5971 MSD mass spectrometer, scan time 1/ sec., directly coupled to a HP 5890 gas chromatograph, using a J & W DB-5, 0.26 mm x 30 m, 0.25 micron coating thickness, fused silica capillary column (see Adams, 2007 for operating details). Identifications were made by library searches of the Adams volatile oil library (Adams, 2007), using the HP Chemstation library search routines, coupled with retention time data of authentic reference compounds. Note that limonene and β-phellandrene elute as a single peak on DB-5, but their amounts can be quantitated by the ratio of masses 68, 79 (limonene) and 77, 93 (β-phellandrene). Quantitation was by FID on an HP 5890 gas chromatograph using a J & W DB-5, 0.26 mm x 30 m, 0.25 micron coating thickness, fused silica capillary column using the HP Chemstation software.

RESULTS AND DISCUSSION

The volatile leaf oils of *Pinus eldarica (*and *P. brutia)* contain considerable amounts of β-pinene (20.7%), α-pinene (11.3%), (E)-caryophyllene (13.3%) and germacrene D (9.2%) with lesser concentrations of δ-3-carene (2.5%), limonene (3.4), β-phellandrene (2.2), (E)-β-ocimene (3.2), α-terpinyl

acetate (2.6), α-humulene (2.4), phenyl-ethyl-3-methyl-butanoate (2.6), and (E)-γ-bisabolene (2.1). Cultivated *P. eldarica* trees (USA, Utah) had a similar profile dominated by germacrene D (27.4%), α-pinene (14.5%), β-pinene 13.4%) and (E)-caryophyllene (10.5%) and with moderate amounts of δ-3-carene (4.5%), limonene (0.9), β-phellandrene (1.3), (E)-β-ocimene (1.6), α-terpinyl acetate (2.3) and α-humulene (2.1).

The oil from samples from the natural stand in Azerbaijan differs quantitatively in some compounds from that of the cultivated *P. eldarica* in the USA (both were extracted and analyzed by identical methods). This is apparent (Table 2) in the concentrations of α-pinene (11.3%, 14.5%), β-pinene (20.7,13.4), δ-3-carene (2.5, 4.5), limonene (3.4, 0.9), β-phellandrene (2.2, 1.3), (E)-caryophyllene (13.3, 10.5), and germacrene D (9.2, 27.4).

The oil analysis from Iran (Sulriman and San'aty (2005) was generally quite similar to that from Azerbaijan and USA, but differed in having some unusual components (red, Table 2): citronellol, citronellyl formate, elemicin, and geranyl isovalerate. These compounds, usually minor, may have come from cross-contamination with an essential oil containing these compounds.

Analyses (from the literature) for plants from Italy (red, Table 2) differ considerably from Azerbaijan in some components (cf. β-pinene (1.4%, Italy), α-terpinyl acetate (54.3%, Italy), germacrene D (missing, Italy), along with several other components missing in the Italy analysis of Vidrich et al. 1999. The high amount of α-terpinyl acetate (54.3%) is inconsistent with other oils of *P. eldarica* (Table 2), and seems to be spurious.

The question of the distinctness of *P. eldarica* and *P. brutia* (ssp. *brutia*) is addressed in the oil analyses as we have included two analyses of *P. brutia* from a native, Lebanon stand, and cultivated in Greece in Table 2. Several compounds separate *P. eldarica* and *P. brutia* (green and yellow, Table 2) including α-pinene, camphene, β-pinene, myrcene, (Z)-β-ocimene, α-campholenal, trans-sabinol, camphor, α-terpinyl acetate, (E)-caryophyllene, germacrene D, germacrene D-4-ol, α-cadinene and sandaracopimarinal. Several of these are qualitative differences, but most of these are in low concentrations and may have been found (or reported) in other analysis in *P. brutia*. Still, the overall pattern is clear that the oils of *P. eldarica* and *P. brutia* do differ in several components, and this seems to support the practice of recognizing *P. eldarica* (or as P. *brutia* ssp. *brutia*) in Azerbaijan and Georgia, where these taxa are part of the native flora.

A comparison of the oils of individuals of *P. eldarica* cultivated in St. George, Utah, USA, revealed considerable variation with 3 to 5 fold differences between lowest and highest values (Table 3): α -pinene (6.2 - 19.7%); α -pinene (5.0 - 24.3%); δ-3-carene (1.1 - 7.6%); terpinolene (0.2 - 2.5%); α-terpinyl acetate (1.6 - 3.3%); (E)-caryophyllene (6.6 - 19.4%) and germacrene D (21.6 - 39.0%). This large amount of variation reflects the origin of all *P. eldarica* (Mondell pine, etc.) in the US that were obtained by growing seedlings from the USDA bulk lot of seeds from Afghanistan in 1961. It is very possible that the Afghanistan *P. eldarica* might have actually come from Persia originally, and the Persian *P. eldarica* pines were likely from Azerbaijan (or less likely, Georgia).

There is a suggestion that two chemotypes (or races) might exist in *P. eldarica* as one can see that several trees have a similar oil profile (15576, 15579, 15575, blue, Table 3) with higher α-pinene, α-pinene and δ-3-carene and trees 15578, 1577 and 1574)yellow, Table 3) are higher in germacrene D and (E)-caryophyllene. Additional sampling is needed to confirm if chemical races exist in nature.

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Table 2. The leaf oil constituents of *Pinus eldarica* from a natural, endemic population in Azerbaijan, compared with *P. eldarica* cultivated in the USA, Italy and Iran. Also included are analyses of the volatile leaf oils from the closely related (conspecific?) species, *P. brutia*. Compounds that differ in amounts between *P. eldarica* and *P. brutia* are highlighted in yellow.

KI	compound	*P. eldarica* natural, Azerbaijan	*P. eldarica* cult. USA. U St. George	*P. eldarica* cult. Iran Isfahan[1]	*P. eldarica* cult. Italy[2]	*P. brutia* natural, Lebanon[3]	*P. brutia* cult. Greece[4]
921	tricyclene	t	t	-	-	t	-
924	α-thujene	t	0.1	-	-	t	t
932	**α-pinene**	**11.3**	**14.5**	**11.8**	**12.8**	**18.9**	**16.0**
946	**camphene**	**0.2**	**0.2**	**0.2**	**0.2**	**0.8**	**0.8**
948	benzaldehyde		-	-	2.1	-	-
969	sabinene	t	0.3	-	t	-	0.9
974	**β-pinene**	**20.7**	**13.4**	**7.9**	**1.4**	**31.2**	**45.7**
988	**myrcene**	**1.1**	**0.9**	**0.7**	**0.6**	**2.1**	**2.4**
1002	α-phellandrene	t	t	t	-	0.1	t
1008	δ-3-carene	2.5	4.5	1.7	-	2.4	0.5
1014	α-terpinene	t	0.1	t	t	0.1	0.1
1020	p-cymene	t	t	-	t	t	-
1024	limonene	3.4	0.9	3.2(w β-phell?	4.0	4.0	1.6
1025	β-phellandrene	2.2	1.3	-	0.2	2.5	1.1
1044	**(Z)-β-ocimene**	**0.1**	**0.4**	**0.3**	-	-	-
1044	(E)- β-ocimene	3.2	1.6	1.1	-	1.5	1.9
1054	γ-terpinene	0.1	0.1	0.1	0.1	0.2	0.3
1086	terpinolene	0.6	0.8	0.5	0.1	1.0	1.3
1095	linalool	0.1	0.4	0.1	0.3	0.2	0.7
1114	endo-fenchol	t	0.1	-	-	0.1	0.1
1122	α-campholenal	t	t	-	-	-	-
1136	trans-sabinol	t	t	-	-	-	-
1141	**camphor**	**0.1**	**0.1**	**-**	**-**	**-**	**-**
1165	borneol	t	0.1	-	-	-	0.1
1174	terpinen-4-ol	0.1	0.1	t	-	0.1	0.5
1179	p-cymen-8-ol	-	-	-	0.2	-	-
1186	α-terpineol	0.8	0.1	0.2	0.2	-	1.2
1195	myrtenol	t	t	t	0.1	-	-
1218	endo-fenchyl acetate	-	-	-	-	-	0.4
1227	citronellol	-	-	0.3	-	-	-
1247	linalool acetate	-	-	-	-	2.3	0.2
1273	citronellyl formate	-	-	0.3	-	-	-
1284	bornyl acetate	0.5	0.2	0.4	1.0	0.4	-
1342	trans-piperitol acetate	-	0.1	-	-	-	-
1345	**α-terpinyl acetate**	**2.6**	**2.3**	**3.8**	**54.3**	-	0.8
1374	α-copaene	0.1	0.1	0.3	0.2	0.4	-
1375	geranyl acetate	-	-	-	-	0.5	0.3
1387	β-bourbonene	0.1	0.6	3.3	0.8	0.5	-
1389	β-elemene	t	t	-	-	0.2	-
1398	methyl eugenol	t	t	-	-	0.3	-
1400	β-longipinene	0.4	0.1	0.3	-	0.2	-
1417	**(E)-caryophyllene**	**13.3**	**10.5**	**17.1**	**12.5**	**5.9**	**4.9**
1430	β-copaene	t	0.2	-	-	-	-
1454	α-humulene	2.4	2.1	4.2	2.2	1.5	0.9
1464	9-epi-(E)-caryophyllene	0.4	0.3	t	-	-	-
1478	γ-muurolene	t	t	-	1.3	-	-
1480	**germacrene D**	**9.2**	**27.4**	**26.6**	**-**	**14.5**	**7.6**
1480	phenyl-ethyl-3-me-butanoate	2.6	0.3	-	0.8	-	0.8
1495	γ-amorphene	0.3	0.2	-	-	-	-
1491	methyl isoeugenol	t	t	-	-	0.8	-
1500	α-muurolene	0.3	0.2	-	t	0.5	-
1500	(E,E)-α-farnesene	-	-	-	-	0.4	-
1511	δ-amorphene	0.3	0.2	0.5	-	-	-
1513	γ-cadinene	0.2	0.5	0.7	0.5	1.9	-
1522	δ-cadinene	0.5	1.3	1.7	1.9	-	0.4
1529	(E) γ -bisabolene	2.1	-	-	-	-	-

KI	compound	*P. eldarica* natural, Azerbaijan	*P. eldarica* cult. USA. U St. George	*P. eldarica* cult. Iran Isfahan[1]	*P. eldarica* cult. Italy[2]	*P. brutia* natural, Lebanon[3]	*P. brutia* cult. Greece[4]
1537	α-cadinene	-	-	0.2	-	-	-
1555	elemicin	-	-	4.3	-	-	-
1574	**germacrene-D-4-ol**	**0.2**	**0.6**	-	-	-	-
1583	caryophyllene oxide	1.3	0.4	1.4	-	0.3	-
1608	humulene epoxide II	0.3	t	-	-	-	-
1608	β-atlantol	0.3	t	-	-	-	-
1610	geranyl isovalerate	-	-	1.8	-	-	-
1638	epi-α-cadinol (= T-cadinol)	t	t	0.3	-	-	-
1640	phenyl ethyl hexanoate	-	0.2	-	-	-	-
1640	**epi-α-muurolol**	**t**	**0.1**	**-**	**0.9**	**-**	**-**
1652	**α-cadinol**	**0.5**	**0.5**	**0.9**	**-**	**-**	**-**
1710	pentadecanal	0.6	t	-	-	-	-
1890	sesquiterpene alcohol?, 43,79,6 192, FW 222?	0.9	-	-	-	-	-
1958	iso-pimara-8(14),15-diene	-	t	-	-	-	-
1987	manool oxide	0.6	t	-	-	-	-
2184	**sandaracopimarinal**	**0.3**	**0.3**	**-**	**-**	**-**	**-**
2200	docosane (C22)	t	t	-	-	-	-
2300	tricosane (C23)	t	0.2	-	-	-	-
2400	tetracosane (C24)	t	0.3	-	-	-	-
2443	methyl neoabietate	t	0.2	-	-	-	-

[1]Suleiman and San'aty (2005), [2]Vidrich et al. (1999), [3]Ghosn and Saliba (2006), Roussis et al. (1995). KI = Kovat's Index (linear), t = trace, < 0.05%.

Table 3. Variation in composition among six *P. eldarica* trees oil., cultivated, using drip irrigation, in St. George, Utah, USA. Components never larger than a trace (t) are omitted.

KI	compound	*P. eldarica 15576*	*P. eldarica 15579*	*P. eldarica 15575*	*P. eldarica 15578*	*P. eldarica 15577*	*P. eldarica* cult. USA *15574*	*P. eldarica* cult. USA. Range
921	tricyclene	t	0.1	t	t	t	t	t - 0.1
924	α-thujene	t	t	t	t	t	0.1	t - 0.1
932	**α-pinene**	**19.7**	**13.2**	**19.1**	**12.6**	**15.6**	**6.2**	**6.2 - 19.7**
946	camphene	0.3	0.2	0.3	0.2	0.2	0.1	0.1 - 0.3
969	sabinene	0.2	0.1	0.1	0.7	t	t	t -0.7
974	**β-pinene**	**24.3**	**22.2**	**15.0**	**5.0**	**10.8**	**5.0**	**5.0 - 24.3**
988	myrcene	1.0	1.0	1.0	0.9	0.7	0.6	0.6 - 1.0
1002	α-phellandrene	t	t	t	t	t	0.1	t - 0.1
1008	**δ-3-carene**	**7.5**	**7.3**	**2.9**	**6.3**	**1.1**	**0.6**	**1.1 - 7.5**
1014	α-terpinene	0.1	t	t	0.1	0.1	t	t - 0.1
1020	p-cymene	0.1	t	t	t	t	t	t - 0.1
1024	limonene	0.7	1.2	1.0	0.7	0.5	1.3	0.5 - 1.3
1025	β-phellandrene	1.1	1.7	1.4	1.0	0.8	2.0	0.8 - 2.0
1044	(Z)-β-ocimene	0.3	0.4	0.5	0.6	0.2	0.4	0.2 - 0.6
1044	(E)- β-ocimene	1.0	1.0	4.0	1.4	0.9	1.1	1.0 - 4.0
1054	γ-terpinene	0.1	0.1	0.1	0.1	0.1	0.1	0.1 - 0.1
1086	**terpinolene**	1.1	1.1	**0.6**	**1.5**	**0.3**	**0.2**	**0.2 - 1.5**
1095	linalool	0.7	0.4	0.3	0.7	t	0.1	t - 0.7
1114	endo-fenchol	t	0.1	t	t	t	t	t - 0.1
1136	trans-sabinol	t	0.3	t	t	t	t	t - 0.3
1141	camphor	t	0.2	t	t	t	t	t - 0.3
1165	borneol	t	0.2	t	t	t	t	t - 0.2
1174	terpinen-4-ol	t	0.2	t	0.1	t	t	t - 0.2
1186	α-terpineol	0.2	0.1	0.1	t	0.1	0.1	t - 0.2
1195	myrtenol	t	0.1	t	t	t	t	t - 0.1
1284	bornyl acetate	0.2	0.4	0.3	0.2	t	0.4	t - 0.4
1342	trans-piperitol acetate	0.1	0.2	0.1	0.1	t	0.1	t - 0.2
1345	**α-terpinyl acetate**	**2.2**	**2.6**	**2.7**	**3.3**	**1.6**	**2.4**	1.6 - 3.3
1374	α-copaene	t	0.1	0.1	0.1	0.2	0.1	t - 0.2
1387	β-bourbonene	0.6	1.1	0.3	0.8	0.4	0.8	0.3 - 1.1
1389	β-elemene	t	t	t	t	t	0.1	t - 0.1
1400	β-longipinene	0.4	0.2	0.3	0.3	0.1	0.2	0.1 - 0.4
1417	**(E)-caryophyllene**	**6.6**	**8.8**	**8.2**	**12.0**	**16.0**	**19.4**	**6.6 - 19.4**
1430	β-copaene	0.1	0.2	0.1	0.2	0.2	0.3	0.1 - 0.3
1454	α-humulene	1.3	1.7	1.6	2.4	3.1	3.9	1.3 - 3.9
1464	9-epi-(E)-caryophyllene	0.1	0.1	t	0.3	0.1	1.1	t - 1.1
1478	γ-muurolene	t	t	0.1	t	t	t	t - 0.1
1480	**germacrene D**	**21.7**	**21.6**	**28.5**	**37.1**	**35.2**	**39.0**	**21.6 - 39.0**
1480	**phenyl-ethyl-3-me-butanoate**	**1.9**	**0.9**	**1.0**	**0.9**	**0.6**	**0.6**	**0.6 -1.9**
1495	γ-amorphene	0.2	0.3	0.3	0.4	0.4	0.9	0.2 - 0.9
1500	α-muurolene	0.2	0.2	0.2	0.3	0.3	0.5	0.2 - 0.5
1511	δ-amorphene	0.1	0.1	0.3	0.1	0.3	0.5	0.1 - 0.5
1513	γ-cadinene	0.2	0.4	0.5	0.5	0.6	1.1	0.2 - 1.1
1522	δ-cadinene	0.3	1.0	1.4	1.2	1.6	2.8	0.3 - 2.8
1574	germacrene-D-4-ol	0.6	0.6	0.6	1.0	0.7	0.9	0.6 - 1.0
1583	caryophyllene oxide	0.4	0.4	0.3	0.5	0.3	0.5	0.3 - 0.5
1608	humulene epoxide II	t	0.1	t	t	t	t	t - 0.1
1608	β-atlantol	t	0.1	t	t	t	0.1	t - 0.1
1638	epi-α-cadinol (= T-cadinol)	t	t	t	0.1	t	t	t - 0.1
1640	phenyl ethyl hexanoate	0.2	t	t	0.1	0.2	0.2	t - 0.2
1640	epi-α-muurolol	0.1	t	t	t	0.1	t	t - 0.1
1652	α-cadinol	0.3	0.5	0.5	0.5	0.5	0.7	0.3 - 0.7
1958	**iso-pimara-8(14),15-diene**	**t**	**0.5**	**0.6**	**0.2**	**0.4**	**0.2**	**t - 0.6**
1987	manool oxide	t	t	t	t	t	0.1	t - 0.1
2184	**sandaracopimarinal**	**t**	**0.7**	**0.9**	**0.1**	**0.1**	**t**	**t - 0.9**
2200	docosane (C22)	t	t	t	t	t	0.5	t - 0.5
2300	**tricosane (C23)**	**t**	**t**	**t**	**t**	**0.2**	**0.6**	**t - 0.6**
2400	tetracosane (C24)	0.3	0.3	0.2	0.5	0.3	0.2	0.2 - 0.5
2443	methyl neoabietate	t	0.2	0.2	0.2	0.3	0.1	t - 0.3

Screening hydrocarbon yields of sunflowers: *Helianthus maximiliani, H. grosseserratus H. nuttallii,* and *H. tuberosus* in the North Dakota-Minnesota-South Dakota area

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ABSTRACT

Analyses of biomass (g DW 10 leaves), % HC yields, and g HC yield/ g DW 10 leaves for four perennial sunflower species, *Helianthus grosseserratus, H. maximiliani, H. nuttallii ssp.* ssp. *rydbergii,* and *H. tuberosus* revealed that these taxa are all low in biomass in Minnesota, North Dakota, and South Dakota. Percent HC yields were lower (*H. grosseserratus* 5.42, *H. maximiliani* 3.18, *H. nuttallii ssp.* ssp. *rydbergii* 4.37, *H. tuberosus* 2.97%) than in annual, *H. annuus* in Texas. The small amount of biomass coupled with low % HC resulted in meager HC yields (g HC/ g DW leaves). However, a few high HC yielding plants were found: 7.76% *H. nuttallii*; 5.97% H. *grosseserratus*; 4.40% *H. tuberosus*; and 4.95% *H. maximiliani*.
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KEY WORDS: *Helianthus maximiliani, H. nuttallii,* Sunflower, yields of hexane soluble leaf hydrocarbon.

There has been considerable interest in bio-renewable sources of hydrocarbons (HC). Sunflowers have been surveyed on several occasions for hydrocarbons. Adams and Seiler (1984) surveyed 39 taxa of sunflowers for their cyclohexane (hydrocarbon) and methanol (resins) concentrations and reported the highest cyclohexane (bio-crude) yielding taxa were *H. agrestis*, an annual, Bradenton, FL (7.38%) and *H. annuus*, Winton, OK (7.09%). Further work by Adams et al. (1986) examined 614 plant taxa from the western US for their hydrocarbon (hexane soluble) and resin (methanol soluble) yields. They reported 2 plants of *H. annuus* from Idaho with 8.71% and 9.39% hydrocarbon yields. Recently, the survey of *H. annuus* was greatly expanded throughout the northcentral and southwestern US (Adams et al. 2017a; Adams et al. 2018a). Figure 1 (from Adams, et al. 2018a) shows the highest yielding plants were found in the Texas Panhandle (7.99%) with considerable geographical variation in % HC yields.

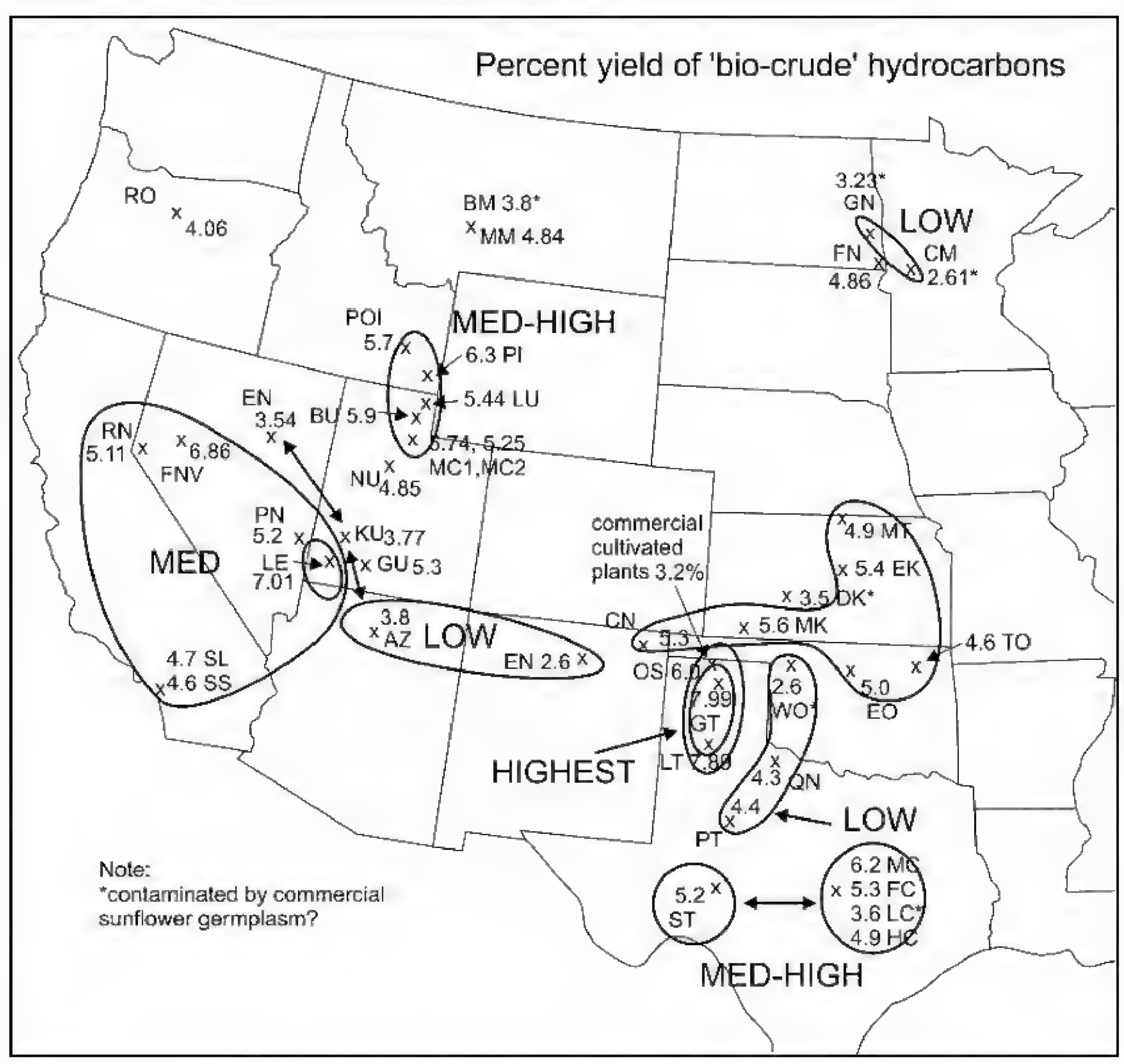

Figure 1. Geographic variation in the % HC in *H. annuus* (from Adams et al. 2018a).

Adams and Seiler (1984) surveyed 39 taxa of sunflowers that were grown in a common garden at the USDA lab, Bushland, TX. They analyzed cyclohexane (hydrocarbon), rubber, methanol (resins) yields plus protein concentrations. They reported cyclohexane (HC) yields for *H. maximiliani* of 3.10 - 3.50%; *H. nuttallii* 5.25% - 5.17%; *H. grosseserratus* 2.36 - 4.41% and *H. tuberosus* 2.26% (Table 1).

The purpose of the present study was to extensively examine variation in biomass, % HC yields and g HC/ biomass (g wt. 10 leaves) of four perennial sunflower species, *Helianthus grosseserratus, H. maximiliani, H. nuttallii ssp.* ssp. *rydbergii,* and *H. tuberosus* from a small geographic area in the North Dakota, Minnesota, South Dakota region. This report is a continuation of surveys on HC and rubber, and studies on the induction of HC in sunflowers (Adams and TeBeest, 2016; Adams et al. 2016, 2018c; Adams et al. 2017b).

Table 1. Analyses of 39 Taxa of *Helianthus*, representing 49 accessions. Modified from Adams and Seiler, 1984).

Helianthus species	Annual (A) or perennial (P)	Origin	C-hex fract.	Rubber yield[a]	MeOH fract.	Total	Percent Protein[b]
agrestis	A	Bradenton, FL	7.38	1.66	13.45	20.83	6.9
angustifolius	P	Alvin, TX	3.33	0.18	9.58	12.91	15.9
annuus	A	Winton, OK	7.09	1.40[c]	11.73	18.82	8.7
annuus, hybrid 894	A	Bushland, TX	2.23	0.49	14.65	16.88	8.6
anomalus	A	Mexican Water, AZ	5.74	0.18	12.30	18.04	9.8
agrophyllus	A	Rockport, TX	6.52	1.14[c]	9.60	16.12	11.9
arizonensis	P	Snowflake, AZ	6.13	0.28[c]	13.16	19.29	18.4
californicus	P	Napa, CA	3.05	1.78[c]	12.44	15.49	13.8
ciliaris	P	Bushland, TX	5.26	0.57	17.17	22.43	15.6
debilis	A	Titusville, FL	1.95	0.68	8.83	10.78	9.6
deserticola	A	Leeds, UT	3.16	0.82	10.96	14.12	5.3
divaricatus	P	Wister, OK	1.09	0.47[d]	11.54	13.44	2.6
glaucophyllus	P	Blowing Rock, NC	3.29	0.25	9.50	12.79	8.1
grosseserratus	**P**	**Cherokee Co., KS**	**2.36**	**0.28**	**12.28**	**14.64**	**14.6**
grosseserratus	**P**	**Hooker Co., KS**	**4.41**	**0.28**	**14.37**	**18.78**	**20.1**
grosseserratus	**P**	**Stuart, OK**	**3.56**	**0.28**	**10.49**	**14.05**	**17.1**
hirsutus	P	Wilburton. OK	1.60	0.30	8.30	9.90	6.1
laciniatus	P	Mimbres River, NM	3.15	0.31	12.40	15.55	9.9
laetiflorus	P	Lyon Co., KS	2.22	0.66	10.64	12.86	11.9
laevigatus	P	Botetourt Co., VA	3.53	na	18.24	21.77	13.9
maximiliani	**P**	**Bloomington, IN**	**3.10**	**na**	**13.21**	**16.31**	**10.8**
maximiliani	**P**	**San Jon, NM**	**3.50**	**0.24**	**9.87**	**13.37**	**15.3**
maximiliani	P	Gatesville, TX	2.53	na	10.30	12.83	8.9
microcephalus	P	Cherokee Co., SC	4.77	0.26[c]	14.25	19.02	14.1
mollis	P	Greenwood Co., KS	3.26	0.31	11.05	14.31	8.9
mollis	P	Okmulgee Co., OK	2.60	0.31	9.72	12.32	8.5
mollis	P	Rivercrest, TX	1.87	0.31	8.58	10.45	6.6
neglectus	A	Kermit, TX	3.83	0.10	11.71	15.54	16.2
nuttallii	**P**	**Orovada, NV**	**5.25**	**0·96[c]**	**10.23**	**15.48**	**8.8**
nuttallii	**P**	**Payson, UT**	**5.17**	**na**	**12.76**	**17.93**	**10.6**
occidentalis	P	Raymondville, MO	2.12	0.48	15.14	17.26	11.9
occid. ssp. *plantagineus*	P	Sheridan, TX	2.36	1.62	18.33	20.69	8.8
paradoxus	A	Ft. Stockton, TX	3.46	0.15	19.54	23.00	13.3
petiolaris ssp. *fallax*	A	Adrian, TX	2.15	0.30	11.99	14.14	17.3
petiolaris ssp. *petiolaris*	A	Memphis, TX	1.86	0.14	21.00	22.86	12.1
praecox ssp. *hirtus*	A	Carrizo Springs, TX	5.19	0.49	10.05	15.24	13.8
pumilus	P	Boulder, CO	1.72	0.53	6.87	8.59	7.4

resinosus	P	Collins, MS	2.89	1.78[c]	11.76	14.65	11.9
rigidus ssp. *rigidus*	P	Brookston, IN	1·86	na	9.93	11.79	7.8
rigidus ssp. *subrhomboides*	P	Leyden, CO	1·42	na	10.90	12.32	9.9
salicifolius	P	Kansas	3.13	0.37	9.30	12.43	7.1
salicifolius	P	Muenster, TX	3.26	0.37	9.31	12.57	11.2
silphioides	P	Wister, OK	2.63	0.42	18.01	20.64	10.0
simulans	P	Milton, FL	3.42	0.31	13.91	17.33	18.1
smithii	P	Morgantown, NC	4.48	0.58[c]	11.77	16.25	12.2
strumosus	P	Siler City, |NC	2.98	0.55	11.80	14.78	12.9
tuberosus	**P**	**Kilgore, TX**	**2.26**	**0.93**	**13.28**	**15.54**	**12.1**
tuberosus x *annuus*	P	Turlock, CA	1.73	na	12.21	13.94	9.3
Average			3.39	0.57	12.26	15.65	11.35

a Rubber yields, for leaves except for *divaricatus,* are from Stipanovic et al. 1980. *b* Protein determined by Kjeldahl N x 6-25. *c* By 13C·NMR spectral analysis. All others by gravimetric. *d* Whole plant analyzed.

MATERIALS AND METHODS

Population locations - see Appendix I. Ten or sometimes 20 mature leaves were collected at stage R 5.1- 5.3, that is, when first flower head has opened with mature rays. Leaves were air dried in paper bags at 49° C in a plant dryer for 24 hr or until 7% moisture was attained. Leaves were ground in a coffee mill (1mm). 3 g of air-dried material (7% moisture) were placed in a 125 ml, screw cap jar with 20 ml hexane, the jar sealed, then placed on an orbital shaker for 18 hr. The hexane soluble extract was decanted through a Whatman paper filter into a pre-weighed aluminum pan and the hexane evaporated on a hot plate (50°C) in a hood. The pan with hydrocarbon extract was weighed and tared. Raw yields were corrected by a correction factor (CF) that was developed from data obtained by performing a soxhlet, 6 hr extraction. The correction factor = soxhlet hexane yields divided by yields from 18 hr shaking in hexane = 2.06. Analysis of variance (ANOVA) and SNK (Student-Newman-Keuls) multiple range tests were performed based on the formulations in Steel and Torrie (1960).

RESULTS

Analyses of biomass (g dw 10 leaves), % HC yields and g HC yield/ 10 leaves are shown for 17 populations of *H. maximiliani* in Table 2. Biomass is small due to the small leaves and ranged from 3.37 g / 10 lvs. (Moorhead, MN) to only 1.54 g (Milnor, ND). The % HC yields were not very large and varied from 3.85% (x 12. Harwood, ND) to 2.23% (x 15, Oaks, ND, Fig. 2). There was not much of a regional trend in HC yields (Fig. 2), so perhaps the variation was mostly due to edaphic factors, rather than genetic factors.

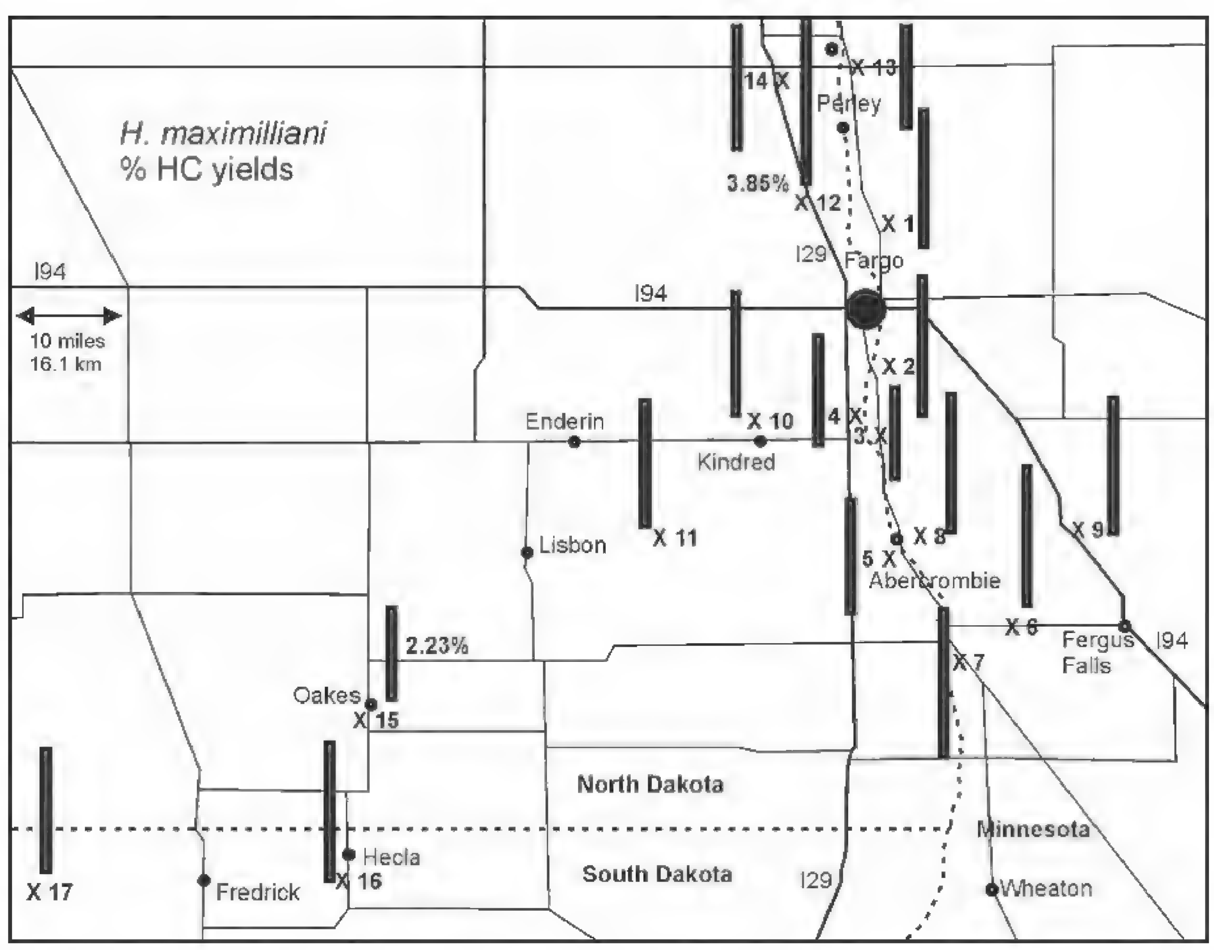

Figure 2. Distribution of % HC yields for *H. maximiliani.*

Table 2. The yields biomass (g dw, 10 lvs), % HC yield, g HC/ g 10 lvs for *H. maximiliani*. Any two means in a column with the same suffix letter are **not** significantly different (P= 0.05).

H. maximiliani popn. IDs	g wt / 10 lvs	% HC yield	Coef. of var. %	Range of yields	g HC/ plant (10 lvs)	Site information
ANOVA F ratio, signif. Probability, P	15.6*** $3.7x10^{-7}$	7.1*** $6.2x10^{-5}$			16.1*** $3.0x10^{-7}$	
12 Harwood, ND	2.28cd	**3.85m**	7.06	3.40-4.22	0.088y	North of Harwood, ND, dry roadside ditch near RR tracks, ~40 plants near some trees.
M7 Milnor, ND	1.54f	3.59mn	4.90	3.30-3.81	0.056yz	West of Milnor, ND, dry roadside ditch next to soybean field, ~50 scattered plants.
M2 Comstock, MN	1.85cdef	3.41mno	19.40	2.33-4.94	0.062yz	North of Comstock, MN, dry undisturbed roadside ditch, next to soybean field, 20-30 plants.
M6 DeLamere, ND	1.79cdef	3.40mno	5.44	3.09-3.61	0.061yz	South of DeLamere, ND, dry roadside ditch next to soybean field, 30 plants.
M8 Lisbon, ND	1.88cdef	3.38mno	8.02	3.09-3.81	0.064y	East of Lisbon, ND, dry roadside ditch next to soybean field, 40-50 plants.
16 Hecla, SD	2.23bcde	3.38mno	7.63	2.98-3.71	0.076y	South of Hecla, SD, dry roadside ditch but seasonally moist, 20 plants.
M9 McLeod, ND	2.32cd	3.34mno	5.33	2.99-3.50	0.078y	Northwest of McLeod, ND, dry roadside ditch, scattered population of ~100 plants.
M1 Moorhead, MN	**3.37a**	3.32mno	12.98	2.74-4.12	0.112x	North of Moorhead, MN, roadside ditch along RR tracks, dry undisturbed area, 100 scattered plants.
11 Sheldon, ND	1.82cdef	3.11mnop	13.67	2.37-3.40	0.056yz	South of Sheldon, ND, dry roadside ditch next to corn field, 30-40 scattered plants.
10 Kindred, ND	2.2cd8	2.99nop	6.59	2.78-3.30	0.068y	West of Kindred, ND, Upper dry slopes of roadside drainage ditch, 50 plants.
14 Kelso, ND	2.04cdef	2.99nop	4.33	2.78-3.19	0.061yz	South of Kelso, ND, seasonally moist roadside ditch, ~100 plants scatter in ditch.
17 New Effington, SD	2.21bcde	2.98nop	6.12	2.78-3.30	0.066y	East of New Effington, SD, roadside ditch near powerline R/W, seasonally moist cut over area, ~200 plants
M5 Abercrombie ND	1.99cdef	2.81nopqr	12.70	2.40-3.43	0.113x	North of Abercrombie, ND, dry roadside ditch next to soybean field, 75 scattered plants.
M4 Lithia, ND	2.69b	2.72opqr	7.65	2.40-3.02	**0.145w**	North of Lithia, ND, dry roadside ditch next to sunflower field, 25 plants
13 Hendrum, MN	2.17bcde	2.51pqr	6.14	2.37-2.78	0.055yz	North of Hendrum, MN, dry roadside ditch near old RR track, ~ 100 scattered plants.
M3 Wolverton, MN	2.74b	2.28qr	26.27	1.51-3.18	0.064y	North of Wolverton, MN, dry undisturbed roadside ditch along fence row next to soybean field, 50-60 plants.
15 Oaks, ND	1.64ef	2.23r	6.28	2.06-2.37	0.037z	West of Oaks, ND, dry roadside ditch, ~150 plants.
Results from other *H. maximiliani* populations (from Adams et al. 2018b)						
MXOK	2.43	**5.06**	26.8	3.16-7.82	0.123	15333, *H. maximiliani,* Langston, OK, K. Hart
MXMT	1.43	3.71	9.5	3.32-4.47	0.053	15276, *H. maximiliani,* Bozeman, MT M. Lavin, wet cattail area, dry in summer
MxMC	**4.98**	3.68	24.7	2.54-5.42	**0.180**	15342, *H. maximiliani,* McLennan Co., TX W. Holmes
MxCC	4.25	3.24	30.0	2.17-5.42	0.138	15340, *H. maximiliani,* Coryell Co., TX W. Holmes,
MxFC	4.04	2.20	24.3	1.19-3.17	0.089	15341, *H. maximiliani,* Falls Co., TX W. Holmes,

Some of the populations were quite variable with a COV (Coefficient Of Variation) that ranged from 26.27% (Wolverton, MN, x 3) to a low of 4.33% near Kelso, ND (x 14, Table 2). The largest % yield was a plant with 4.94% in the Comstock, MN (x 2) population. Yields of HC, as g/ g DW 10 leaves, varied from 0.145 (x 4), Lithia, ND down to 0.037 (x 15), Oaks, ND (Table 2).

High HC yielding plants of *H. maximiliani* (from Adams et al. 2018b) are shown at the bottom of Table 2 for comparisons. The Langston, OK population (Table 2) has a % HC yield of 5.06% that is much higher than found in this study. However, the other *H. maximiliani* populations sampled have somewhat lower HC yields. The McLennan Co. TX population had high biomass (4.98g) and a high HC g wt yield (0.180, Table 2).

Analyses of % HC yields of *H. nuttallii* ssp. *rydbergii* populations (Fig. 3) revealed that highest yield was from the Kindred, ND area (x 3, 6.54%) and the lowest yield, 2.61% was nearby (x 2). The population near Kindred (x 3) appears to be the most unusual population sampled in having larger % HC yields. This may be due to some type of microhabitat at that site.

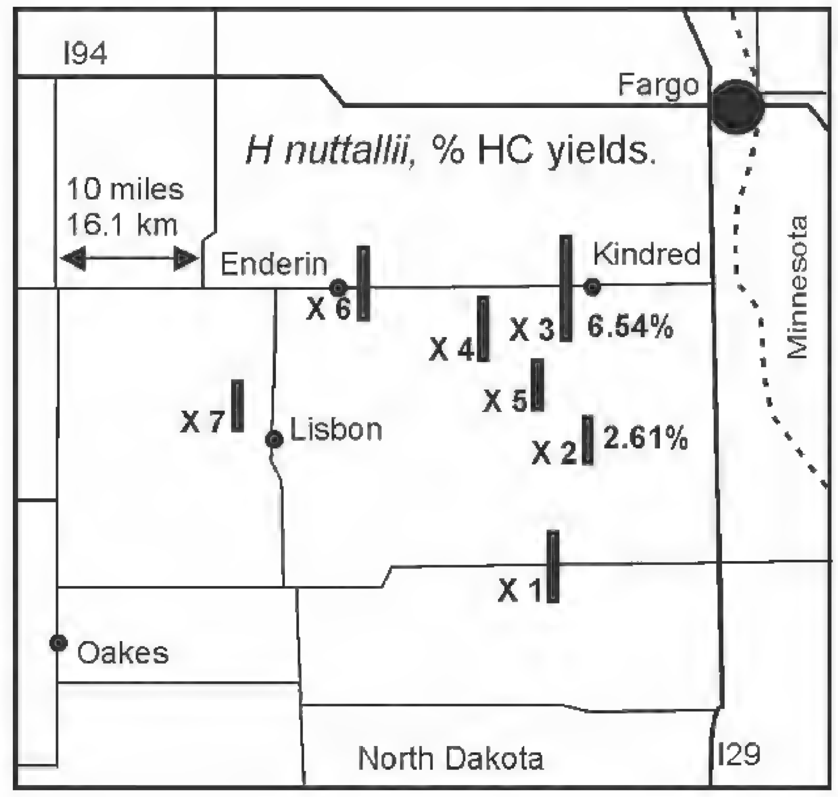

Figure 3. % HC yields for *H. nuttallii*.

Table 3. The yields biomass (g dw, 10 lvs), % HC yield, and g HC/ g 10 lvs for *H. nuttallii* ssp. *rydbergii*. Any two means in a column with the same suffix letter are **not** significantly different (P= 0.05).

H. nuttallii ssp. *rydbergii* popn. IDs	g wt / 10 lvs	% HC yield	Coef. of var. %	Range of yields	g HC/ plant (10 lvs)	Site information
ANOVA F ratio, signif. Probability, P	 78.1*** 1.4×10^{-6}	 53.5*** 3.8×10^{-7}			 16.0*** 3.4×10^{-4}	
N3 Kindred, ND	2.05d	**6.54**g	11.08	5.71-**7.76**	**0.134**x	Southwest of Kindred, ND, moist swampy roadside ditch near the Sheyenne National Grasslands, large scattered population of ~200 plants.
N6 Enderlin, ND	2.42bc	5.21h	6.92	4.64-5.77	0.127x	East of Enderlin, ND, swampy roadside ditch, very large population of ~300 scattered plants.
N1 DeLamere ND	1.32f	5.01h	6.14	4.78-5.62	0.067z	South of DeLamere, ND, moist swampy roadside ditch next to soybean field, 30 plants.
N4 Leonard, ND,	2.67c	4.53i	10.86	3.91-5.15	0.103xy	South of Leonard, ND, swampy roadside ditch near sunflower field, very large scattered population of ~500 plants.
N5 Lisbon, ND	2.72b	3.56j	8.29	3.09-3.91	0.098y	West of Lisbon, ND, swampy roadside ditch, scattered population of 30-40 plants.
N7 Marion, ND	1.34e	3.55j	5.20	3.30-3.82	0.047z	Southwest of Marion, ND, swampy roadside ditch, edge of cattail slough, large population extending ½ mile.
N2 Wyndmere ND	**4.14**a	2.61k	8.60	2.27-2.88	0.108xy	North of Wyndmere, ND, roadside ditch moist swampy area, near the Cheyenne National Grasslands, scattered population of ~75-100 plants.
Results from other *H. maximiliani* populations (from Adams et al. 2018b)						
NuGUT	**2.23**	**7.02**	16.4	5.58-**9.16**	**0.156**	15260, *H. nuttallii*, Glendale UT Adams, wet ditch, dry in summer

NuSAZ	2.03	5.43	9.5	4.49-6.07	0.110	15290, *H. nuttallii,* Licher, Sedona, AZ,
NuKUT	1.75	3.12	37.7	1.7-5.26	0.055	15263, *H. nuttallii,* Kanab, UT Adams wet bank of spring fed pond.

Analyses of % HC yields of *H. tuberosus* populations (Fig. 4) revealed that highest yield was from north of Perley, MN (x 6, 3.79%) and the lowest yield was from x 2 (1.98%, Fig. 4). The higher yielding populations are grouped around Perley. The highest yielding individual, 4.40%, was from the Perley (x 6) population.

It might be noted that the single population of *H. grosseserratus*, in South Dakota (Gros, Fig. 4), was quite high in % HC (5.42%). The coefficient of variation in % HC yields was not large (several populations from 11.50 - 16.51% (Table 4).

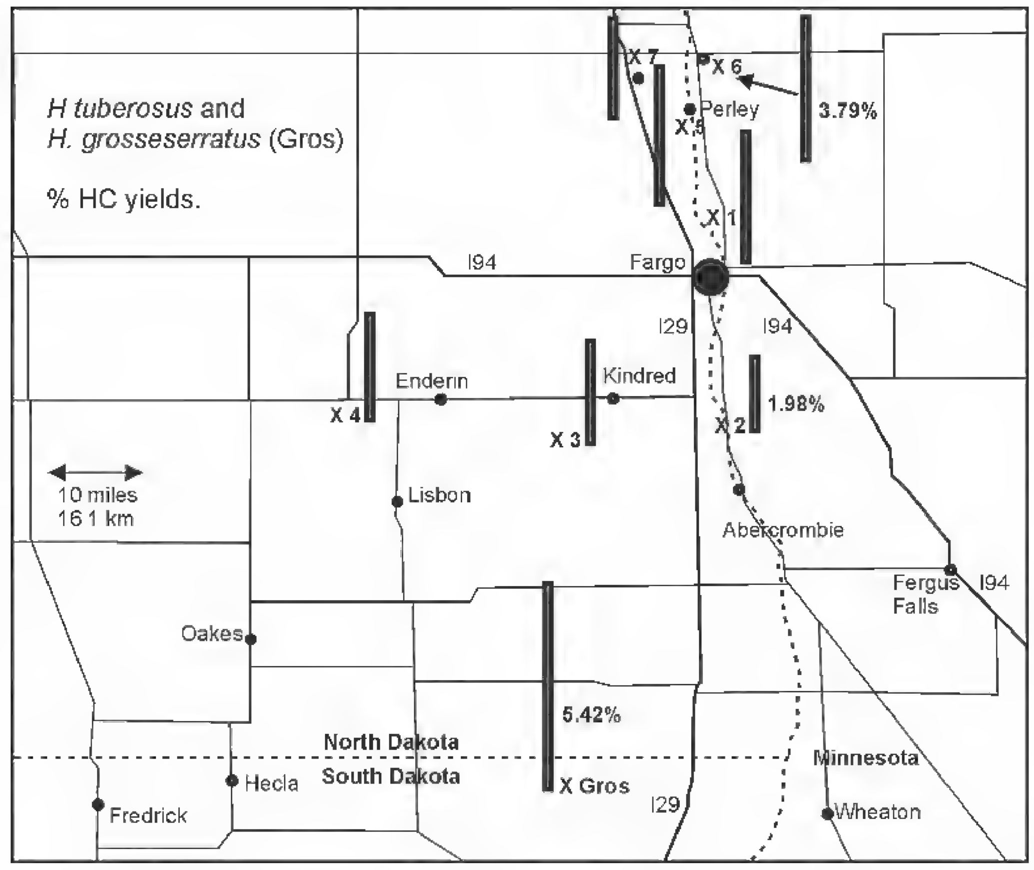

Figure 4. Distribution of % HC yields of H. *tuberosus* and a single population of *H. grosseserratus* (Gros).

Leaf biomass was considerably larger for *H. tuberosus* due to larger leaves and ranged from 9.50 down to 4.98 g/ g DW 10 leaves (Table 4). This is approximately 3 times larger than found in *H. maximiliani*, or *H. nuttallii*. This resulted in very large yields (north of Perley, 0.294 and east of Perley 0.261 g/ g DW 10 leaves, Table 4). *Helianthus tuberosus* (Jerusalem artichoke) can be cultivated for inulin in the tubers that can be converted to fructose and thence to ethanol (Sachs et al. 1981). Jerusalem artichoke can also produce a significant amount of above ground biomass that can be converted to fuels by cellulosic digestion (Duvniak et al. 1991). Seiler (1993) reported forage yields of Jerusalem artichoke (JA) cultivars grown in Texas sampled at flowering varied from 3.0 to 6.3 mg ha^{-1}, with the cultivated JA 'Sunchoke' having the highest yield at 6.3 Mg/ha, while at maturity, cultivar 'Vadim' had the highest mean tuber yield of 9.04 mg/ha, making it a potential multi-purpose biofuel crop.

We also compared the biomass and HC yields for the taxa surveyed in this study with some populations of *H. annuus* from previous studies (Table 5). Notice that among the four taxa in this study, biomass was significantly larger in *H. tuberosus*, but % HC yield and g HC/ plant were the highest in *H. grosseserratus* based on a single population.

Examination of the biomass and yields from *H. annuus* from 3 high yielding populations (Table 5) shows that their biomass was 2.5 to 5 times as large as that of *H. maximiliani, H. nuttallii, H. tuberosus* and *H. grosseserratus*. The % HC yields from *H. annuus* from natural populations were large (5.75 - 7.99 %) and correspondingly, the g HC/ g 10 vs. was much higher than the four perennial taxa in this study because they have mostly relatively small leaves.

Table 4. The yields biomass (g dw, 10 lvs), % HC yield, and g HC/ g 10 lvs for *H. tuberosus* and *H. grosseserratus*. Any two means in a column with the same suffix letter are **not** significantly different (P= 0.05).

H. tuberosus popn. IDs	g wt / 10 lvs	% HC yield	Coef. of var. %	Range of yields	g HC/ plant (10 lvs)	Site information
ANOVA F ratio, signif. Probability, P	 42.3*** 7.8×10^{-6}	 15.9*** 2.6×10^{-4}			 19.0*** 1.6×10^{-4}	
T6 north Perley, MN	7.13c	**3.79**g	12.13	3.09-4.40	**0.261**x	North of Perley, MN, swampy area, flood plain of Wild Rice River, small popn. 30 plants.
T5 east Perley, MN	8.20b	**3.63**g	3.93	3.43-3.78	**0.294**x	East of Perley, MN, moist river bank of the Red River of the North near bridge, ~45 plants.
T1 Moorhead, MN,	5.60de	**3.46**g	16.51	2.78-4.33	0.197y	North of Moorhead, MN, roadside ditch near bridge of drainage ditch, moist area along edge of trees, 40-50 plants.
T4 Enderlin, ND	6.10d	2.84h	8.17	2.47-3.09	0.174y	East of Enderlin, ND, swampy roadside ditch, small scattered population of 30 plants.
T3 Kindred, ND	5.55de	2.74h	13.43	2.34-3.30	0.150yz	Southwest of Kindred, ND, swampy roadside ditch near sunflower field, large population ~200 plants scattered for several hundred feet.
T7 Kelso, ND	4.98e	2.36hi	4.65	2.20-2.54	0.118z	North of Kelso, ND, moist roadside with 50 plants scattered along the edge of trees.
T2 Christine, ND	**9.50**a	1.98i	11.50	1.72-2.34	0.186y	East of Christine, ND, roadside ditch near bridge of the Red River of the north, moist bank area along edge of trees, ~200 plants.
***H. grosseserratus*, Veblen, SD**						
GRO, Veblen, SD	5.35	**5.42**	5.19	4.94-5.97	**0.292**	East of Veblen, SD, seasonally moist roadside drainage area with cattails, scattered population on both sides of road, ~100 plants

Table 5. The yields of biomass (g dw, 10 lvs), % HC yield, and g HC/ g 10 lvs for *H. maximiliani, H. nuttallii, H. tuberosus* and *H. grosseserratus* compared with *H. annuus* (from Adams et al. 2017b). Any two means in a column with the same suffix letter are **not** significantly different (P= 0.05).

Species	biomass g wt / g wt 10 lvs	% HC yield	g HC/ plant (10 lvs)
ANOVA: F ratio, significance Probability, P =	147.7*** 9.5×10^{-10}	39.4*** 7.2×10^{-7}	107.7*** 3.8×10^{-9}
H. maximiliani	2.20c	3.18i	0.076z
H. nuttallii	2.31c	4.37h	0.095z
H. tuberosus	**6.72**a	2.97i	0.197y
H. grosseserratus	5.35b	**5.42**g	**0.292**x
***H. annuus*, natural vs. seeds from that population grown in greenhouse**			
H. annuus, ex natural Gruver, TX	**19.36**	**7.99**	**1.499**
H. annuus, greenhouse plants grown from seed from Gruver, TX	2.54 **(13.1%)**	3.67 **(45.9%)**	0.092 **(6.1%)**
H. annuus, natural Lake Tanglewood, TX	**19.11**	**7.88**	**1.484**
H. annuus, , greenhouse plants grown from seed from Lake Tanglewood, TX	2.79 **(14.6%)**	4.38 **(55.6%)**	0.120 **(8.1%)**
H. annuus, natural Salt Lake City, UT	**11.92**	5.75	0.672
H. annuus, greenhouse plants grown from seed from Salt Lake City, UT,	2.69 **(22.6%)**	4.50 **(78.3%)**	0.121 **(17.9%)**

However, one of the perplexing factors in *H. annuus* is the strong environmental component present when plants are grown in a greenhouse. Notice that % HC yields decreased in the greenhouse grown plants. In fact, % HC yields were only 45.9, 55.6, and 78.3% in the greenhouse grown vs. naturally grown plants (Table 5). The Texas populations (Gruver, TX, Lake Tanglewood, TX) in the Texas Panhandle were very stressed with wilted leaves and leaves eaten by grasshoppers and other insects. It may be that higher HC production was induced in these populations. At present, research involving stressing greenhouse plants by the application of growth regulators have not revealed any chemical that can induce high HC yields (Adams and Johnson, 2018; 2019; Adams et al. 2016; 2017b).

Finally, it should be noted that *H. maximiliani, H. nuttallii, H. tuberosus* and *H. grosseserratus* are grow in seasonally wet to swampy habitats (see Appendix I, Site information). But, *H tuberosus* can be cultivated in drier soils, so it may have some potential as a multi-product crop.

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Appendix I. Locations of populations of *H. maximiliani, H. grosseserratus, H. nuttallii and H. tuberosus* sampled in this study.

H. maximiliani popn. IDs	samples/ popn.	Site information	County	Latitude	Longitude
M1 MAX MM, Moorhead, MN	9	North of Moorhead, MN, roadside ditch along RR tracks, dry undisturbed area, 100 scattered plants.	Clay, MN	N 46.97702	W 96.74728
M2 B3 MAX CM, Comstock, MN	9	North of Comstock, MN, dry undisturbed roadside ditch, next to soybean field, 20-30 plants.	Clay, MN	N 46.73838	W 96.76250
M3 B4 MAX WM, Wolverton, MN	5	North of Wolverton, MN, dry undisturbed roadside ditch along fence row next to soybean field, 50-60 plants.	Wilkin, MN	N 46.59403	W 96.74290
M4 B6 MAX LtN, Lithia, ND	5	North of Lithia, ND, dry roadside ditch next to sunflower field, 25 plants	Richland, ND	N 46.63033	W 96.82085
M5 B7 MAX AN Abercrombie, ND	5	North of Abercrombie, ND, dry roadside ditch next to soybean field, 75 scattered plants.	Richland, ND	N 46.45640	W 96.73614
M6 B9 MAX DN, DeLamere, ND	5	South of DeLamere, ND, dry roadside ditch next to soybean field, 30 plants.	Sargent, ND	N 46.23442	W 97.32183
M7 B10 MAX MLN, Milnor, ND	5	West of Milnor, ND, dry roadside ditch next to soybean field, ~50 scattered plants.	Sargent, ND	N 46.22578	W 97.54917
M8 B11 MAX LbN, Lisbon, ND	5	East of Lisbon, ND, dry roadside ditch next to soybean field, 40-50 plants.	Ransom, ND	N 46.44221	W 97.63865
M9 B12 MAX McN, McLeod, ND	5	Northwest of McLeod, ND, dry roadside ditch, scattered population of ~100 plants.	Ransom, ND	N 46.44226	W 97.32230
10 B14 MAX KN, Kindred, ND	5	West of Kindred, ND, Upper dry slopes of roadside drainage ditch, 50 plants.	Cass, ND	N 46.62954	W 97.07226
11 B19 MAX SN, Sheldon, ND	5	South of Sheldon, ND, dry roadside ditch next to corn field, 30-40 scattered plants.	Ransom, ND	N 46.47187	W 97.48933
12 B22 MAX HN, Harwood, ND	5	North of Harwood, ND, dry roadside ditch near RR tracks, ~40 plants near some trees.	Cass, ND	N 46.97702	W 96.89918
13B25 MAX HM, Hendrum, MN	5	North of Hendrum, MN, dry roadside ditch near old RR track,~ 100 scattered plants.	Norman, MN	N 47.30009	W 96.81122
14B27 MAX KN, Kelso, ND	5	South of Kelso, ND, seasonally moist roadside ditch, ~100 plants scatter in ditch.	Traill, ND	N 47.30401	W 97.02623
15 B29 MAX ON, Oaks, ND	5	West of Oaks, ND, dry roadside ditch, ~150 plants.	Dickey, ND	N 46.13889	W 98.14533
16 B30 MAX HS, Hecla, SD	5	South of Hecla, SD, dry roadside ditch but seasonally moist, 20 plants.	Brown, SD	N 45.79483	W 98.14483
17 B32 MAX NES, New Effington, SD	5	East of New Effington, SD, roadside ditch near powerline R/W, seasonally moist cut over area, ~200 plants	Roberts, SD	N 45.84889	W 96.89655

H. nuttallii popn. IDs	samples/ popn.	Site information	County	Latitude	Longitude
N1 B8 NUT (*nuttallii* ssp. *rydbergii*)	5	South of DeLamere, ND, moist swampy roadside ditch next to soybean field, 30 plants.	Sargent, ND	N 46.22195	W 97.32242
N2 B13 NUT (*nuttallii* ssp. *rydbergii*)	5	North of Wyndmere, ND, roadside ditch moist swampy area, near the Sheyenne National Grasslands, scattered population of ~75-100 plants.	Richland, ND	N 46.39833	W 97.13411

N3 B15 NUT (*nuttallii* ssp. *rydbergii*)	5	Southwest of Kindred, ND, moist swampy roadside ditch near the Sheyenne National Grasslands, large scattered population of ~200 plants.	Richland, ND	N 46.58162	W 97.13760
N4 B17 NUT (*nuttallii* ssp. *rydbergii*)	5	South of Leonard, ND, swampy roadside ditch near sunflower field, very large scattered population of ~500 plants.	Richland, ND	N 46.56163	W 97.21896
N5 B18 NUT (*nuttallii* ssp. *rydbergii*)	5	West of Lisbon, ND, swampy roadside ditch, scattered population of 30-40 plants.	Richland, ND	N 46.45131	W 97.21825
N6 B20 NUT (*nuttallii* ssp. *rydbergii*)	5	West of Enderlin, ND, swampy roadside ditch, very large population of ~300 scattered plants.	Ransom, ND	N 46.62957	W 97.52242
N7 B28 NUT (*nuttallii* ssp. *rydbergii*)	5	Southwest of Marion, ND, swampy roadside ditch, edge of cattail slough, large population extending ½ mile.	LaMoure, ND	N 46.48585	W 98.15190

H. grosseserratus popn. IDs					
G1 B31 (*grosseserratus*)	10	East of Veblen, SD, seasonally moist roadside drainage area with cattails, scattered population on both sides of road, ~100 plants.	Marshall, SD	N 45.86354	W 97.32122

H. tuberosus popn. IDs	samples /popn.	Site information	County	Latitude	Longitude
T1 B1 TUB MM, Moorhead, MN	5	North of Moorhead, MN, roadside ditch near bridge of drainage ditch, moist area along edge of trees, 40-50 plants.	Clay, MN	N 46.94864	W 96.77061
T2 B5 CN, Christine, ND	5	East of Christine, ND, roadside ditch near bridge of the Red River of the north, moist bank area along edge of trees, ~200 plants.	Richland, ND	N 46.57238	W 96.75393
T3 B16 TUB KdN, Kindred, ND	5	Southwest of Kindred, ND, swampy roadside ditch near sunflower field, large population of ~200 plants scattered for several hundred feet.	Richland, ND	N 46.57271	W 97.14014
T4 B21 TUB EN, Enderlin, ND	5	East of Enderlin, ND, swampy roadside ditch, small scattered population of 30 plants	Barnes, ND	N 46.63010	W 97.87658
T5 B23 TUB ePM, Perley, MN	5	East of Perley, MN, moist river bank of the Red River of the North near bridge, ~45 plants.	Norman, MN	N 47.17957	W 96.82209
T6 B24 TUB nPM, Perley, MN)	5	North of Perley, MN, swampy area, flood plain of Wild Rice River, small population of 30 plants.	Norman, MN	N 47.28012	W 96.81380
T7 B26 TUB KsN, Kelso, ND	5	North of Kelso, ND, moist roadside with 50 plants scattered along the edge of trees.	Traill, ND	N 47.32582	W 97.03096

Populus acuminata Rydb., hybrid or hybrid-derived species?

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ABSTRACT

Populus acuminata Rydb. has mostly been considered to be a hybrid between *P. angustifolia* E. James and varieties of *P. deltoides* W. Bartram ex Marshall or *P. fremontii* S. Watson depending on location. Several populations of *P. acuminata* were observed for about 15 years for any seed production. Seed production did not occur in most years because the catkins were frozen by late spring freezes. Seed was finally obtained from one population, germinated, and grown in an unheated greenhouse for 2 years. First year leaves resembled *P. angustifolia* but second year leaves matched the parent *P. acuminata*. Seedling establishment rarely occurs because no seeds are produced or conditions are too dry for establishment in most years. Results of this study support recognizing *Populus acuminata* as a hybrid-derived species.
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KEY WORDS: *Populus acuminata,* Salicaceae, hybrid-derived species

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INTRODUCTION

Populus acuminata Rydb. (Salicaceae) has been interpreted by some workers as a hybrid (BONAP 2019; Eckenwalder 2010; USDA, NRCS 2019) and by others as a hybrid-derived species (Cronquist 1964, Dorn 2001). One probable parent is *P. angustifolia* E. James (Figure 1) and the other is either (1) *P. deltoides* W. Bartram ex Marshall var. *occidentalis* Rydb. [ssp. *monilifera* (Aiton) Eckenw.] (Figure 1) at the type locality and at other locations where that variety occurs, or (2) *P. deltoides* var. *wislizeni* (S. Watson) Dorn where that variety occurs, or (3) *P. fremontii* S. Watson [*P. deltoides* var. *fremontii* (S. Watson) Cronquist] where that species occurs (Crawford 1974, Jones & Seigler 1975). It has also been observed that *P. acuminata* occurs in places where one or both probable parents do not occur (Cronquist 1964, Eckenwalder 1984, Cronquist & Dorn 2005). Crawford (1974) discussed the many problems regarding the status of *P. acuminata*. This study adds more data toward resolution of the problems.

METHODS

Several populations of *P. acuminata* were observed for about 15 years for any seed production. These populations included Carter Canyon, the type locality, and North Platte National Wildlife Refuge in Scotts Bluff County, Nebraska, Fort Laramie National Historic Site and Wyoming Game & Fish Rawhide Wildlife Unit in Goshen County, Wyoming, and Cottonwood Draw and North Platte River at Guernsey in Platte County, Wyoming. Twelve seeds were collected from catkins on one individual plant in Platte County, Wyoming, were germinated on a wet coffee filter in a petri dish, and grown in pots in an unheated greenhouse near Lingle, Wyoming for 2 years. All plants of *P. acuminata* in the population where the seeds were collected were measured at breast height for circumference which was then converted to diameter at breast height (DBH). This population is about 87 km from the type locality.

RESULTS AND DISCUSSION

The populations of *P. acuminata* that were observed were all interspersed with plants of *P. deltoides* var. *occidentalis*. The other probable parent of *P. acuminata*, *P. angustifolia*, was not present at most of the sites. Seed production did not occur in *P. acuminata* in most years because either the staminate catkins, or the pistillate catkins, or both, were frozen by late spring freezes. When seed production was finally

observed in a population along a dry wash (Cottonwood Draw) about 19 km northeast of Wheatland, Platte County, Wyoming, there was no seedling establishment because conditions were too dry. These observations along with the DBH measurements (Table 1) indicate that seedling establishment only occurs rarely, generally decades apart. Seedling establishment is likely somewhat more frequent at sites where the population is adjacent to a perennial stream.

Of the 12 seeds that were collected, 9 germinated, were transferred to pots of soil, and placed in an unheated greenhouse. Six of the plants survived their first growing season. A representative leaf from the first growing season is shown in Figure 2. It unexpectedly resembles a leaf of *P. angustifolia* with a short petiole and narrow blade. Four of the plants from the first growing season survived the winter. Representative leaves from about midlength of the second year's growth in July are shown in Figure 2 for each of the four plants. These leaves are all typical for *P. acuminata* with the longer petiole and broader blade. A specimen from the *P. acuminata* parent tree (Dorn 12006, MO, RM) and the type specimen (Rydberg 372, NY) are shown in Figure 3 for comparison. Figure 4 shows the four plants grown from seed. The closest known plants of *P. angustifolia* to the population of *P. acuminata* where the seeds were collected is about 14.5 km away near Guernsey, Platte County, Wyoming. The closest known plants of *P. angustifolia* to the type locality are about 50 km away near Lingle, Goshen County, Wyoming. A single plant was found in the seed producing population that suggested a hybrid between *P. acuminata* and *P. deltoides* var. *occidentalis* (Dorn 12195, RM, Figure 5)

The observed reproduction from seed supports a hybrid-derived species. That does not preclude possible extensive hybridization between *P. angustifolia* and *P. deltoides* var. *occidentalis* at other locations. Crawford (1974) concluded that *P. acuminata* had a hybrid origin based primarily on an additive flavonoid profile from the two parents. Jones and Seigler (1975), however, found a flavonoid compound in *P. acuminata* that was not present in the probable parent species. Crawford (1974) did not address the question of whether *P. acuminata* should be considered a hybrid or a hybrid-derived species. It is likely that some plants are F_1 hybrids and others are the result of later seed production and should be considered a hybrid-derived species. Since even the F_1 plants are likely capable of reproducing from seed, perhaps they should all be considered a hybrid-derived species.

The question of whether the plants called *P. acuminata* in the southwestern states where *P. deltoides* var. *occidentalis* does not occur are the same or similar genetically as the more northern and eastern plants is still open. A detailed genetic study might provide insights into that question.

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Table 1. Diameters at breast height (DBH) of *Populus acuminata* plants in the population from which seeds were collected for this study. Seeds were taken from the plant with a DBH of 23.4 cm. The similar DBH's of plants from 17.8 to 23.4 cm and from 79.2 to 88.1 cm suggest two favorable establishment periods. The others suggest establishment with less favorable conditions.

Plant Number	DBH (cm)	Plant Number	DBH (cm)	Plant Number	DBH (cm)
1	4.8	6	23.4	11	88.1
2	17.8	7	34.8	12	99.6
3	19.3	8	64.8	13	118.1
4	21.1	9	79.2	14	127.0
5	22.6	10	84.1	15	139.2


Figure 1. Typical leaves of *Populus angustifolia*, left, and *Populus deltoides* var. *occidentalis*, right, the probable parents of *Populus acuminata*.


Figure 2. Left: Representative leaf from first growing season of plant grown from seed of *Populus acuminata*. Right: Leaves from midlength of second year's growth in mid July from four plants grown from seed of *Populus acuminata*. Scale is 9 cm.

Figure 3. Left: Specimen from *Populus acuminata* parent tree (Dorn 12006). Right: Type specimen of *Populus acuminata* (Rydberg 372, NY).

Figure 4. Four plants grown from seed of *Populus acuminata*, second growing season in mid July.

Figure 5. Probable hybrid between *Populus acuminata* and *Populus deltoides* var. *occidentalis* (Dorn 12195).

Taxonomic novelties in páramo plants. *Espeletia ramosa* (Asteraceae), a new species from Colombia

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ABSTRACT

A new species *Espeletia ramosa* (Espeletiinae, Asteraceae) is described and illustrated. This species is not apparently allied to any other, however its morphological relationships with the most similar *Espeletia* species are discussed. It is currently only known from a small subpáramo area near "Laguna de Tota", Boyacá department, Cordillera Oriental, Colombia. *Espeletia ramosa* is the only Colombian species growing as a compact colony of rosettes branched near the ground, and harbours an array of morphological traits relatively plesiomorphic in comparison with other Espeletiinae members, which make it one of the living species phenotypically closest to the presumed ancestor of the subtribe. In addition, *E. ramosa* represents a notable addition to the diversity of *Espeletia* growth forms in Colombia, a country where *Espeletia* spp. with consistently and profusely branched rosettes were so far unknown. Unfortunately, *E. ramosa* must be considered as an "Endangered" or even "Critically Endangered" species, since its only known population occupies an area < 0.2 km^2. Moreover, the ensemble of areas with relatively undisturbed habitats potentially suitable for the species in the same mountain range likely represents < 10 km^2, is already highly fragmented and seriously threatened by the expansion of the agricultural frontier. Published on-line www.phytologia.org *Phytologia 101(4): 222-230 (Dec 21, 2019).* ISSN 030319430.

KEY WORDS: Andes, Asteraceae, Caulescent Rosette, Colombia, *Espeletia*, Páramo.

The subtribe Espeletiinae (Asteraceae) represents the best example of taxonomic, morphological and ecological diversification in the high elevations of the Northern Andes, particularly in the grassland habitat known as páramo (Cuatrecasas, 1976, 1986, 2013; Diazgranados, 2012; Diazgranados and Barber, 2017; Pouchon et al., 2018; Mavárez, 2019). The ca. 140 species in the group, broadly distributed in the upper Andean forests and páramos of Venezuela and Colombia, with one species in northern Ecuador (*E. pycnophylla* Cuatrec.), have two important diversity centres in the Colombian Cordillera Oriental (ca. 80 spp.) and the Venezuelan Cordillera de Mérida (54 spp.). Espeletiinae exhibit a remarkable degree of morphological diversity, which includes trees with profusely branched, dichotomous or unbranched stems, shrubs, and rosettes that can be sessile, short-branched or, notably, giant caulescent. The latter is a remarkable growth-form that produces an erect stem usually tightly covered by the bases of old and dead leaves, and topped by a crown of green young leaves around the apical bud. The group also exhibits diversity in reproductive strategies; with polycarpic species that reproduce repeatedly across adult life and monocarpic species that reproduce only once before death (Smith, 1981; Cuatrecasas, 2013), and in pollination syndromes; with entomophilous and anemophilous species (Berry and Calvo, 1989). In addition, Espeletiinae are noteworthy by their degree of ecological diversity in regards with tolerance to (a) elevation: ranging from mountain cloud forests at about 1300 m to the very edge of glaciers at 4800 m, (b) humidity: from wet páramo bogs to xeric periglacial talus slopes and rocky outcrops, and (c) solar irradiation: from gaps in the forest to open vegetation such as páramo grasslands. In contrast, these plants show very limited dispersal capacities, as the achenes in all but one species lack pappus (the exception is the scale-like pappus of *E. Chardonii* A.C.Sm.). They are therefore transported by gravity, with long-distance dispersal events hypothesised to have occurred only in a few tree lineages from lower elevations

(Pouchon et al., 2018). Another interesting fact about Espeletiinae is that they frequently become ecologically dominant in the páramos of Northern Ecuador, Colombia and Venezuela in terms of abundance and biomass (Luteyn, 1999). For instance, adult densities of *E. schultzii* Sch.Bip ex Wedd in Venezuela varies between 0.57–2.97 plants/m^2 (Smith, 1981), while local densities of *E. grandiflora* Mutis ex Bonpl in Colombia vary between 0.45–1.43 plants/m^2 (Fagua and González, 2007). Indeed, Espeletiinae usually contribute so largely to the characteristic physiognomy of páramos that for the general public they are the very defining element of this beloved ecosystem, a fact celebrated in countless postcards, books, webpages and even Colombian and Venezuelan national banknotes.

Given the diversity and ecological dominance of Espeletiinae in the páramos, it is not surprising that this group had been subjects of many taxonomic studies. Taxonomic research started with the formal publication of the genus *Espeletia* Mutis ex Bonpl. and the descriptions of three Colombian species (*E. argentea* Bonpl., *E. corymbosa* Bonpl. and *E. grandiflora* Bonpl.) by A. J. A. Bonpland (Humboldt and Bonpland, 1809). Since then, the number of species in the subtribe has been growing considerably, particularly to the extraordinary work due by J. Cuatrecasas (75 spp), but also by S. Díaz-Piedrahita and his collaborators (20 spp), A. C. Smith (9 spp), H. A. Weddell (8 spp), P. C. Standley (6 spp) and L. Aristeguieta (4 spp), among others. One would expect taxonomic research to be nearly complete after more than two centuries of study in a group like Espeletiinae, with its morphological distinctiveness, its association with the comparatively small páramo habitat (ca. 35.000 km^2), and the great attraction they generate to the people, yet the truth is that much taxonomic research remains to be done. For instance, several species are known by only a handful of specimens, while many localities are desperately under-represented or not at all in collections, particularly in Colombia, where access to certain páramos has been hampered by the scarcity of roads and decades of armed conflict.

Here we describe a remarkable new Espeletiinae species from near Laguna de Tota, in the Colombian Cordillera Oriental. The new species was found during a series of fieldtrips set up to some relatively poorly explored páramos in the central section of the Boyacá department in Colombia, a region that nonetheless holds the highest Espeletiinae diversity in the country (Diazgranados, 2012; Cuatrecasas, 2013), and where several new species have been described in the last decade (Díaz-Piedrahita and Rodríguez-Cabeza, 2010).

MATERIALS AND METHODS

Hereafter we follow the classification system for the subtribe Espeletiinae proposed by Mavárez (2019), which considers as taxonomically valid the genus *Espeletia*, while the other genera included in the subtribe by Cuatrecasas (1976, 1995) are treated as heterotypic synonyms of *Espeletia*.

The material described below was collected in August 2019 near el Alto, km 4–5 in the road Pesca-Tota, Boyacá, Colombia (Fig. 1). Most traits were measured on dry samples. However, morphological traits associated with the plant habitus (e.g., stem size and architecture, rosette diameter, number of leaves, number of capitulescences) were measured directly in the field (10 plants). On the other hand, capitulum elements (e.g., phyllaries, disc flowers, ray flowers) were drawn from samples preserved in 50% ethanol. Collected plants of the new species were preserved and distributed to herbarium COL (duplicates will be distributed to herbaria FMB, JBB y UPTC). Collections of somehow similar species already present in other herbaria were also studied (see 'Specimens examined' below). Herbaria acronyms follow Thiers (2019).

TAXONOMIC TREATMENT

Espeletia ramosa Mavárez & Becerra, *sp. nov.*
TYPE: COLOMBIA, Boyacá, near El Alto, about 4.5 km in the road Pesca-Tota, 3060 m., 5.546853 N, -

73.031896 W, August 17 2019, *M.T. Becerra, J. Mavárez* and *J. Aguirre 35* (Holotype: COL; Isotypes: COL). Additional collections from the same type locality (paratypes): *M.T. Becerra, J. Mavárez* and *J. Aguirre 36* (COL), *37* (COL).

Diagnosis. *Espeletia ramosa* is unlike any other species. It is somehow similar to *E. jahnii* Standl., from which it differs by having polycarpic rosettes, much broader leaves, lateral capitulescences and ligulate capitula. It also resembles Colombian *E. pleiochasia* Cuatrec. and *E. garciae* Cuatrec., but differs by having stems profusely branched at ground level, rigidly coriaceous leaves, bracteate capitulescences and ligulate capitula.

Habitus (Fig. 2A): branched polycarpic caulescent rosettes with short, thick stems. Stems are usually divided at ground level into several alternate, prostrate or subterranean branches. Some individuals, usually of small size, appear as undivided and isolated rosettes. Branches usually divided into robust branchlets that can divide again or become terminal, erect and crowned by a rosette. Branches and branchlets are shorter than the rosette diameter, so the latter overlap to a large extent with each other, giving the plant the appearance of a compact colony of up to 15 rosettes. The diameter of terminal branchlets is 7–10 cm at the base of the rosette and their height is frequently smaller than 25 cm, occasionally up to 50 cm. Terminal branchlets are covered by the remains of marcescent leaves and/or their sheaths. Fully grown rosettes are 30–60 cm in diameter, 25–35 cm in height, and are composed by 25–35 green leaves.

Leaf (Fig. 2B, 3A): coriaceous, sessile. Laminae oblanceolate, attenuate towards the base, with acute apex, erect, rigid, total length 23–27 cm, maximum width 4.5–6.5 cm (at 16–18 cm from the base), length to width ratio 3.5–5.7:1, width at mid-length 4.0–5.5 cm, width above base 0.75–1.10 cm. Margins entire, revolute, occasionally undulate. Young leaves loosely covered by a thin veil of light yellow straight hairs up to 4 mm long; becoming paler or whitish, deciduous and shorter with age. Adaxial sides of mature leaves are green, sparsely covered by whitish hairs that are denser and longer near the base, where they can reach up to 4 mm. Very old leaves are almost entirely glabrous adaxially and rough at touch. The costa is visible all along, pale-yellow, mostly flat but prominent at the slightly enlarged base. Abaxial sides of mature leaves are pale-green, covered by a yellowish-creamy indument longer than on the adaxial side and that can reach up to 8 mm near the base. The costa is visible, pale-yellow, round and prominent all along. Secondary nerves mostly parallel but becoming distally irregular (curved or divided), frequently not reaching the leaf margin, adaxially visible although faint, much more prominent abaxially, deviation angles 30°–60°, bases separated by 0.6–1.9 cm at the mid-section of the leaf. Sheaths open, semi-circular, coriaceous, 1.90–2.40 cm long, 3.30–4.40 cm wide, adaxially glabrous, whitish, abaxially woolly, with yellowish hairs up to 1.8 mm long.

Capitulescence (Fig. 2B, C): 1–10 per rosette, coetaneous, corymbose, axillary (lateral), erect, robust, straight or slightly curved, 39–47 cm long, 1.5–2.0x longer than leaves; covered by abundant whitish, villous indument, longer at the capitulescence base, becoming gradually shorter along the axis. Vegetative section 28–34 cm long, ebracteate at the base but with 2–5 alternate bracts along the axis. Reproductive section 8–15 cm long, formed by 9–11 compound branches (i.e., further divided into branchlets), plus several simple branches (undivided) at the top of the axis. Lengths of branches and internodes decrease gradually along the axis, giving the capitulescence the appearance of an almost perfect corymb. Terminal branches and branchlets end with 2–6 capitula.

Capitulum (Fig. 2D, 3B–F): radiate, sub-globbose, small, 6–8 mm in diameter, each with 32–50 flowers, discs 6.5–8.0 mm in diameter, ligular circles 12.5–15.0 mm in diameter. 4–5 external (sterile) phyllaries, thickly herbaceous, adaxially glabrous with 2–5 visible nerves, abaxially villous with hairs < 1 mm long, the outermost pair ovate-triangular, attenuate distally, 3.8–5.2 mm x 2.0–2.2 mm; the others ovate, acuminate, 3.4–5.0 mm x 2.0–3.2 mm. Internal (sterile) phyllaries in two rows, herbaceous, oblong, 3.2–

4.6 mm x 1.6–2.2 mm, adaxially glabrous with 3–4 visible nerves, abaxially villous, hairs < 1 mm long. Fertile phyllaries membranaceous, transparent, oblong, 3.2–3.8 mm x 1.4–1.6, adaxially glabrous, abaxially villous on the distal end. Paleae oblong, 3.1–4.0 mm x 1.2–1.4 mm, distal margin villous. Receptacles convex, glabrous.

Ray flowers (Fig. 3G, H): 10–16 ray flowers per capitulum, in one series. Corollas ligulate; tubes 1.0–1.1 mm long (excluding the ovaries), 0.9–1.0 mm in diameter, densely hirsute, hairs up to 1 mm long; ligules bright yellow, oblong, 3.4–5.2 mm x 1.8–2.5 mm, bi or three-dentate, with a few scattered adaxial hairs. Styles bifid, 1.0–1.5 mm long. Achenes heart-shaped, triangulate, glabrous, dark brown, 1.9–2.1 mm long, 1.1–1.9 m wide. **Disc flowers** (Fig. 3I): 21–36 disc flowers per capitulum, 3.4–4.5 mm long (excluding anthers); tubes 1.8–2.1 mm long, 0.5–0.8 mm in diameter, with a few scattered hairs < 1.0 mm long; limbs 2.0–2.5 mm long when fully open, densely hirsute at the base, hairs up to 1.2 mm long, lobes sparsely hairy; anthers brown, shorter than corolla.
Distribution. Hitherto only known from the type locality.
Ecology. Grows on dry crests, slopes and soils covered by a shrubby sub-páramo vegetation with presence of other rosettes (e.g., *Eryngium*, *Puya*), sclerophyllous shrubs (e.g., *Hesperomeles*, *Monnina*, *Stevia*), herbs (e.g., *Castilleja*, *Halenia*) and some grasses.
Etymology. Refers to the branched stems of the species.
Similar species. Venezuelan *E. jahnii* Standl. also produces branched stems with prostrated or subterranean branches, but their rosettes are monocarpic with strictly linear leaves (length:width ratio 50–100:1), their capitulescences are terminal with profusely bracteate axes, and their capitula are larger (12–18 mm) with eligulate ray flowers. Colombian *E. pleiochasia* Cuatrec. sometimes produces branched caulescent rosettes, but their stems are straight, erect, taller (3–6 m) and divided distally (2–4 rosettes per plant), their oblanceolate leaves are flexible, and their capitulescences are ebracteate. Colombian *E. garciae* Cuatrec. occasionally also produces plants with a branched caulescent rosette habit similar to *E. pleiochasia*, but their capitula are agglomerate and eligulate.

Conservation status. The only known population of *E. ramosa* occupies an area < 0.2 km^2 and is probably composed by a few hundreds of individuals. Its habitat, the shrubby sub-páramo, is seriously threatened by the expansion of the agricultural frontier. The species should for now be included into the IUCN "endangered (EN)" category, until detailed population studies will be done to reach or achieve its conservation status.
Additional specimens examined. ***E. ramosa***: *M.T. Becerra, J. Mavárez & J. Aguirre 36*, *37* (Paratypes: COL). ***E. jahnii***: *J. Cuatrecasas, L. Ruiz-Terán & M. López-Figueiras 28414* (COL), *28414B* (IVIC), *A. Fernández, J. Mavárez, S. Aubert & S. Lavergne 31739* (IVIC), *31759* (IVIC), *J. Cuatrecasas, L. Ruiz-Terán & L. Marcano-Berti 28007* (MERF), *M. López-Figueiras 9149* (MERF). ***E. pleiochasia***: *B. V. Rodríguez-Cabeza, R. Galindo-Tarazona & L. Velasco BVR 2012* (COL), *H. García-Barriga & R. Jaramillo-Mejía 20279* (COL), *L. Uribe-Uribe 1068* (COL), *S. Díaz-Piedrahita 17* (COL), *35* (COL). ***E. garciae***: *L. Uribe-Uribe 6491* (COL), *6491* (COL), *H. García-Barriga & R. Jaramillo-Mejía 20264* (COL), *H. García-Barriga 20369* (COL).

DISCUSSION

Espeletia ramosa is a truly unique species in the subtribe, growing as a compact colony of rosettes branched near the ground. Its habitus is superficially similar to *Espeletia jahnii*, a Venezuelan species that also grows as a compact colony of rosettes branched near the ground or a prostrate shrub. However, *E. ramosa* is different in many important aspects: its rosettes are polycarpic, its leaves are much broader and with less indument, its capitulescences are lateral and its capitula are ligulate. The morphological traits listed above for *E. ramosa* are relatively plesiomorphic in comparison with other members of the genus, which allow us to hypothesize that this new taxon could be one of the living species phenotypically closest to the presumed ancestor of all *Espeletia* (Pouchon et al., 2018). On the

other hand, branching in *E. ramosa* could be character state evolutionarily derived; perhaps acquired through the development of branching in an ancestor with unbranched stems. Indeed, Pouchon et al. (2018) have shown that branching/unbranching are traits relatively plastic in *Espeletia*, which have evolved repeatedly during the history of these plants. The latter suggests that evolutionary inferences based solely on morphological evidence could be misleading in this group, and that formal testing of the alternative scenarios mentioned above will certainly require the analysis of molecular-based phylogenetic reconstructions.

The presence of a constantly and profusely branched rosette in Colombia represents a notable addition to the diversity of *Espeletia* growth forms in this country. Until now, the genus was considered to be represented in Colombia by a large majority of unbranched rosettes, two rosette species in the páramos of Boyacá that may occasionally be sparsely branched, and a few tree species in the north of the Cordillera Oriental, Sierra de Perijá and Sierra Nevada de Santa Marta (Cuatrecasas, 2013; Mavárez, 2019). The much lower diversity of branched forms in Colombia and their geographic proximity to Venezuela motivated the hypothesis of an evolutionary origin for *Espeletia* in Venezuela, with a subsequent dispersal to Colombia, such as suggested by Smith and Koch (1935) and Cuatrecasas (1986, 2013). However, Pouchon et al. (2018) have shown that the radiations of *Espeletia* in Colombia and Venezuela arose rather independently from a common ancestor about 2.5 mya, with relatively recent events of long-distance dispersal between the Andes of the two countries in a few low elevation tree lineages (e.g., *E. neriifolia* (Bonpl. ex Humb.) Sch.Bip. ex Wedd., *E. occulta* S.F.Blake, *E. chardonii*), but not among rosettes. Under the scenario proposed by Pouchon et al. (2018), *E. ramosa* should be phylogenetically closer to other Colombian rosettes, whereas under the scenario of Smith and Koch (1935) and Cuatrecasas (1986), it should be closer to some Venezuelan branched taxa (i.e., *E. jahnii*). However, this matter should be resolved by detailed phylogenetic analyses before a firm statement in this regard can be made.

The demographic and distribution information available indicates that *E. ramosa* must be considered in the IUCN “Endangered (EN)” category. However, although its conservation status of has not been definitely evaluated, preliminary evidence allow us to believe that it could indeed be “Critically endangered (CR)” (IUCN, 2019). First, its only known population occupies an area < 0.2 km^2 and is probably composed by a few hundreds of individuals. On the other hand, the ensemble of areas with relatively undisturbed sub-páramo habitat potentially suitable for the species within the same mountain range likely represents < 10 km^2, is already highly fragmented into a series of small and isolated patches of suitable habitat immersed within a widespread matrix of farmland, and is seriously threatened by the expansion of the agricultural frontier and climate change (Mavárez et al., 2018). In any case, whatever the appropriate threat category for this species, it is clear that some urgent measures are required to guarantee its conservation.

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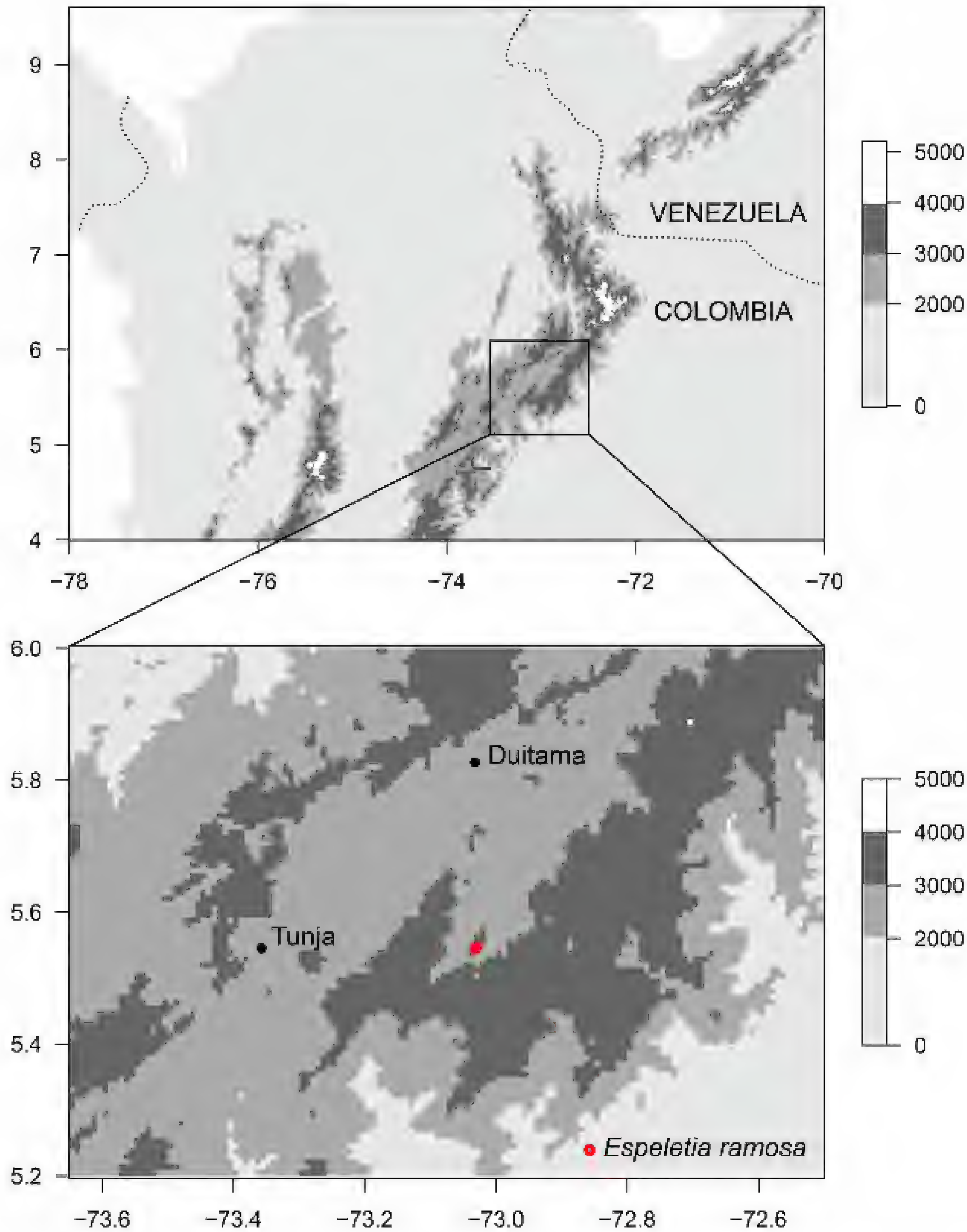

Figure 1. Maps of the Colombian Cordillera Oriental showing the collection locality of *E. ramosa.*

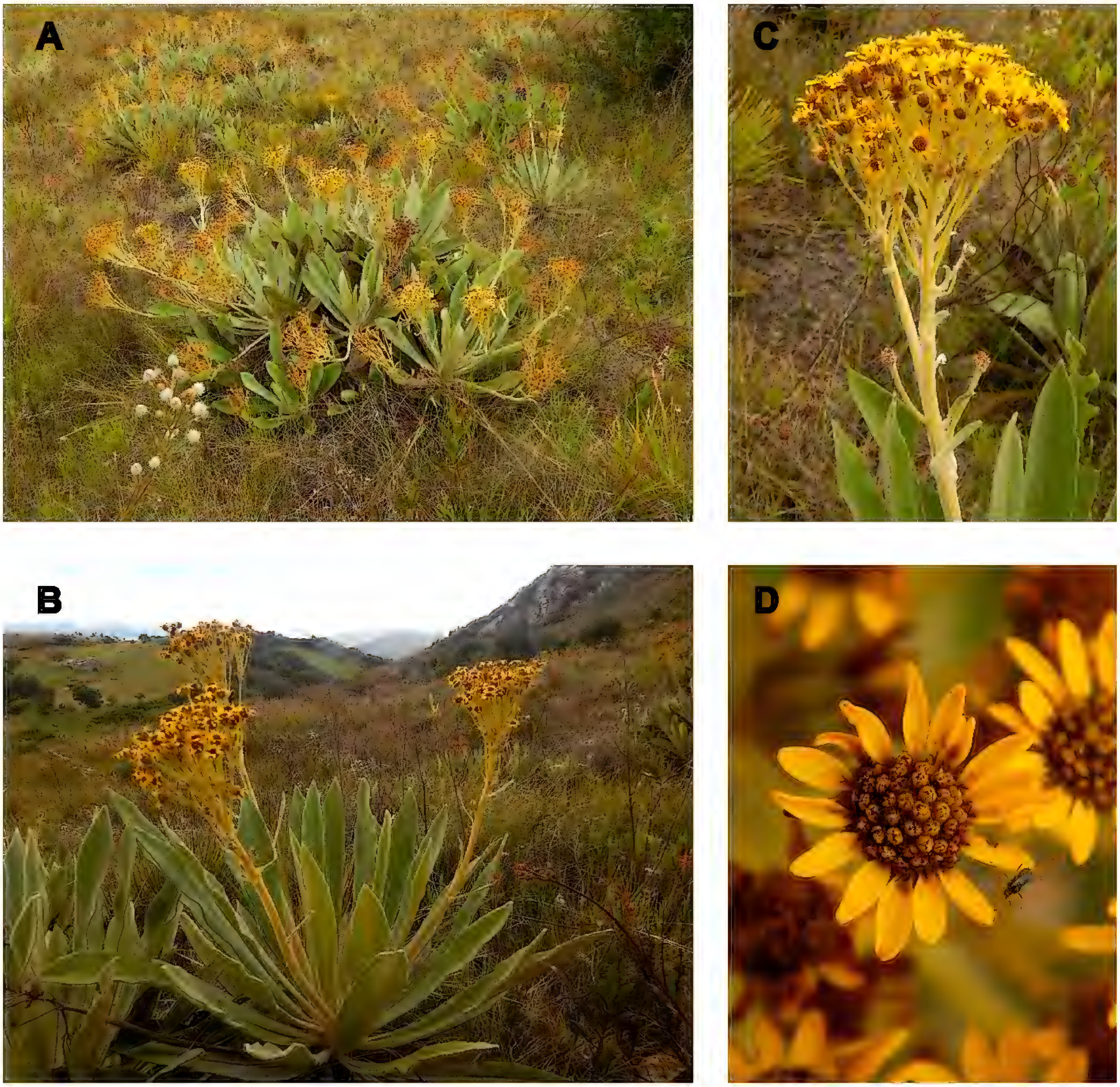

Figure 2. *Espeletia ramosa*. **A**. Example of branched caulescent habit. **B**. Lateral view of individual rosette (note the oblanceolate leaves and alternate bracts along the inflorescence axis). **C**. Lateral view of corymbose inflorescence. **D**. Upper view of capitulum (note the single row and small number of ligulate ray flowers).

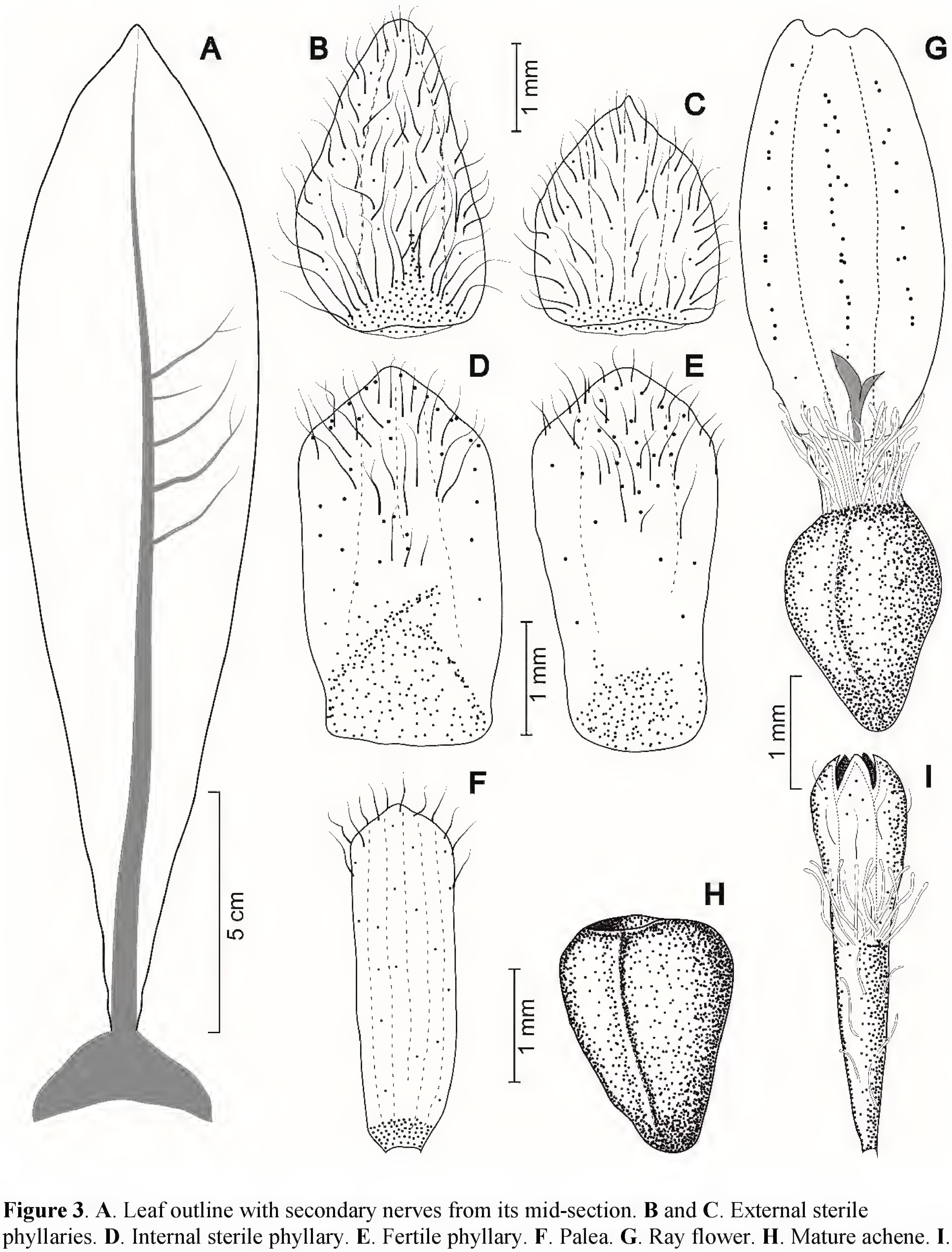

Figure 3. **A**. Leaf outline with secondary nerves from its mid-section. **B** and **C**. External sterile phyllaries. **D**. Internal sterile phyllary. **E**. Fertile phyllary. **F**. Palea. **G**. Ray flower. **H**. Mature achene. **I**. Disc flower (anthers not shown).

Changes in Growth of *Aristida purpurea* Steud. (C_4, Poaceae, Fender's three awn) in the Presence of Arbuscular Mycorrhizal fungi and/or *Solenopsis invicta* Buren (red imported fire ant)

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[2] This manuscript was part of Mr. Engelken's M.S. thesis. Unfortunately, he passed away before it could be published. We are publishing it in his honor. In addition, the thesis document has been edited, references have been updated and the thesis document has been reduced in size. The original document is available at the University of Texas at San Antonio Library.

ABSTRACT

Biomass and percent mycorrhizal infection of roots of *Aristida purpurea* Steud. (C_4 grass, Fender's three awn) changed in the presence of mycorrhizal fungi and/or the red imported fire ant (*Solenopsis invicta* Buren). *Aristida purpurea* dry mass produced and pattern of arbuscular mycorrhizal infection in the roots were examined in heat-sterilized and unsterile soil as well as in various stratified soils. The effectiveness of *S. invicta* as a fungal vector was correspondingly studied. Growth in heat-sterilized soil confirmed that *A. purpurea* is an obligate mycotroph. Percent mycorrhizal infection in non-sterile soil decreased with soil depth despite a uniform distribution of mycorrhizal propagules. Soil stratification altered the pattern of mycorrhizal infection, yet total plant biomass increased when infected roots were able to grow into sterile soil. Growth of *A. purpurea* appeared to be unaffected by delayed initial infection by mycorrhizal fungi as long as upper sterile soil was not more than 20 cm deep. *Solenopsis invicta* does not appear to be a vector of fungal spores and effects of *S. invicta* are subtle and elusive but can seemingly change soil biotic factors so positive growth of *A. purpurea* is maintained. Published on-line www.phytologia.org *Phytologia 101(4): 231-252 (Dec 21, 2019).* ISSN 030319430.

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Both biotic and abiotic factors can affect the growth of plants and ultimately the species composition of communities as well as the rate of community succession (Grace and Tilman 1990; Keddy 2017). There are many biotic factors including symbioses that can affect plant communities and some suggest that over 80% of all terrestrial species have mycorrhizal fungi as symbionts (Smith and Read 2008; Pagano 2012; Willis et al. 2013; Pagano and Grupa 2016). Discovering how these factors interact to influence the growth and distribution of plants is a primary goal of plant ecology (Begon et al. 2006; Keddy 2017).

Mycorrhizae have received attention for their role in affecting plant community composition and roughly 95% of terrestrial plant families have some members known to have mycorrhizal associations (Trappe 1987; Newman 1988; Bush 2008). Mycorrhizae are well known to increase plant growth especially in nutrient poor soils (Gianinazzi 1991; Van Auken and Brown 1998; Van Auken and Fredrick 2006; Bush 2008; Willis et al. 2013; Keddy 2017). Many pines and some orchids, are obligatorily dependent upon mycorrhizae for normal growth and development (Allaby 1992; Keddy 2017). Because mycorrhizae are so prevalent and their potential effects on plant growth so dramatic, it is critical that factors affecting mycorrhizae be examined to determine if relationships are commensal, mutualistic or parasitic.

A symbiotic relationship between the roots of a host plant and a specific soil fungus is a mycorrhizal association (Zak 1964). The presence of mycorrhizae in fossils suggests that this relationship has been coevolving since the Devonian 359-419 million years ago (Gianinazzi 1991). Mycorrhizae are usually mutualistic, absorbing and translocating nutrients (primarily phosphate) to the plant and getting carbohydrates in return (Hayman 1982; Gianinazzi-Pearson and Gianinazzi 1983; Willis 2013).

Mycorrhizae are either ectomycorrhizal (hypha growing between cortical cells) or endomycorrhizae (hypha penetrate root cells) (Peterson and Bonfante 1994; Smith et al. 1994) with ecto's usually associated with roots of woody plants and endo's more typical of non-woody species (Brundrett 1991; Francis and Read 1994). The most prevalent endomycorrhizae are the arbuscular mycorrhizae (Abbott and Gazey 1994) which develop arbuscules where nutrients are exchanged for carbohydrates (Marschner and Dell 1994; Smith et al. 1994; Van Auken and Brown 1998; Van Auken and Fredrick 2006; Bush 2008; Willis et al. 2013; Keddy 2017).

Mycorrhizal propagules are usually most abundant in the upper 50 cm of soil and their numbers decrease with increasing soil depth with few to none occurring at depths below approximately 100 cm (Smith 1978; Zajek et al. 1986). These mycorrhizal fungi have a low degree of host specificity, thus infecting a variety of plants that may lead to the establishment of a network of hyphal interconnections between plants (Read et al. 1985; Francis and Read 1994; Bush 2008; Willis et al. 2013; Keddy 2017). Host dependence upon mycorrhizae can be facultative or obligate but obligate species cannot survive to maturity without mycorrhizae (Miller 1987).

Mycorrhizal dependence was thought related to root morphology (Baylis 1975; Hetrick et al.1990). It has been suggested that C_3 grasses with more branched root systems are less reliant on mycorrhizal associations than C_4 grasses or forbes with coarser, less branched root systems. However, evidence suggested that biomass production in warm-season, C_4 grasses tended to be mycorrhizal dependent while cool-season, C_3 grasses responded less vigorously to mycorrhizae (Hetrick et al. 1988). This has also been attributed to temperature effects (Bentivenga and Hetrick 1992) because C_3 grasses are more common in cooler regions where mycorrhizae would suffer from lower metabolic activity.

Mycorrhizal associations are finely balanced with phenological and edaphic factors known to strongly influence the efficacy of mycorrhizal symbiosis (Brundrett 1991). Of these factors, soil organisms in particular, can influence the occurrence and effectiveness of mycorrhizae. Small mammals (Warner et al. 1987), earthworms (Mcilveen and Cole 1976), and macroarthropods (Visser 1985) have been shown to actively disperse mycorrhizal inoculum altering the distribution and density of propagules (Willis et al. 2013). Sow bugs and millipedes can act as effective vectors by ingesting and dispersing infective mycorrhizal inoculum (Rabatin and Stinner 1988; Wills and Landis 2018).

Because of the widespread abundance of mycorrhizal fungi in soils, they are thought to be an important food source for soil organisms, including nematodes (Hussey and Roncadori 1981) and springtails (Warnock et al. 1982). These soil organisms have been shown to reduce the yield of mycorrhizal plants by grazing on external mycelium. Thus, soil microorganisms may enhance (Fitter and Garbaye 1994), reduce (Hetrick et al. 1990), or not effect (Garbaye 1991) the efficacy of mycorrhizae depending on environmental conditions.

Two insect groups, termites and ants, have long been known for physically modifying soil through selecting, transporting, and rearranging soil particles (Lobry de Bruyn and Conacher 1990), but their potential as vectors for the dispersal of mycorrhizal fungi is not completely known. In addition, the importance of ants in grasslands is often unnoticed and the conversion of over 90% of North American grasslands and savannas to agriculture has put many prairie species at risk of extinction (Wills and Landis 2018). Also, these conversions have changed habitat conditions making many areas susceptible to invasion or encroachment by non-native species (Wills and Landis 2018). Interaction between ants and other species is well known, but complex (Del Toro et al. 2012) and

can alter diversity and community structure (Dostál 2007). Various ant species often reduce predator populations and even the density of parasitoids (Sanders and Van Veen 2011).

A species of ant relatively new to the southern United States including Texas is the red imported fire ant *Solenopsis invicta* Buren (Cook et al. 2016). It arrived in the United states in approximately 1939 and in Texas in 1956 (MacKenzie et al. 2019). There are numerous papers dealing with *S. invicta* management and control (see Woolfolk et al. 2016; MacKenzie et al. 2019; Qin et al. 2019), but little is known of any effects on plant roots or mycorrhizae associated with plant roots. The harvester ant, *Pogonomyrmex occidentalis,* has been shown to assist in the establishment of mutualistic mycorrhizal associations (Freise and Allen 1993; Snyder et al. 2002). However, this is currently unknown for the red imported fire ant. *Solenopsis invicta* has been shown to reduce populations of beneficial predators by as much as 50% (Eubanks et al. 2002).

Solenopsis invicta has continued to increase in abundance and range in North America, but there have been few studies concerning their effects on soil modification, soil organisms, and associated plants (Vinson and Sorensen 1986; Snyder et al. 2002). *Solenopsis invicta* ants are known to feed on the roots of some crop species (Sittle et al. 1983). Concentrations of organic matter and certain mineral nutrients were higher at the surface of *S. invicta* mounds than in the surrounding soil surface. At 15-20 cm depth, only organic matter and potassium were significantly higher in the mounds and average depth of ant activity was 30.0 cm (Lockaby and Adams 1985). *Solenopsis invicta* is known to out compete the native fire ant (*Solenopsis geminate* F.) and replace native colonies at a ratio of 6:1 (Porter et al.1988).

It is possible that pedoturbation by fire ants could result in dispersal of mycorrhizal propagules and changes in plant community structure because they can excavate as much as 1600 kg/ha/yr. But, many of these changes or effects are difficult to detect or observe because they occur below ground (Sanders et al. 2011; Wells and Landis 2018). Nest surface area is small, but below ground area is more extensive making patches or mosaics available for colonization by new and different plant species (Lobry de Bruyn 1999; Boultin and Amberman 2006; Drager et al. 2016). These changes in soil may change microbial activity in and around a nest including actives of soil mycorrhizal fungi, potentially increasing the presence of additional spores and root contact (Dauber et al. 2001, 2008).

PURPOSES

The first purpose of this study was to confirm that growth of the C_4 grass *Aristida purpurea* was mycorrhizal dependent. Second, there was a pattern of mycorrhizal infection and biomass allocation of *A. purpurea* through the vertical soil rhizosphere. Third, could delaying initial infection reduce potential early inhibitory effects of the fungi on the grass? Fourth, what was the potential of *Solenopsis invicta* to act as a vector for spreading mycorrhizal propagules throughout the vertical soil profile?

METHODS

General methods used for all experiments are presented first. All experiments utilized the top 20 cm of a Patrick-series Mollisol. The soil is classified as a clayey-over sandy, carbonatic-thermic, typic calciustoll (Taylor et al. 1966). It was collected near the University of Texas at San Antonio campus in Bexar County, Texas (29°35'N, 98° 40'W). Surface vegetation and litter was removed and the soil sieved through a 6.4 mm mesh sieve, air dried and mixed. A previous soil analysis showed a mean phosphorous content of 12 mg/kg, mean nitrogen content of 1 mg/kg, mean potassium content of 159 mg/ kg, (available form), and a moderately alkaline soil with a pH of 8.4 (Van Auken and Brown 1998). Sterilized soil was sieved, autoclaved at 121°C and 15 ATM for one hr with a 15 min drying period.

In all experiments, the C_4 grass *Aristida purpurea* Steud. (Fender's three awn) was grown from seed in a greenhouse. *Aristida purpurea* is a tufted perennial found on rocky or sandy slopes or in disturbances and is locally abundant in central Texas (Correll and Johnston 1979; USDA-SCS 2019). Seeds of *A. purpurea* were collected from near the campus of the University of Texas at San Antonio in

the spring of 1989 and were stored dry at 4°C until used. For each pot, ten seeds were sown initially by inserting them in the soil such that the awns were the only portions of the seed exposed above the soil surface. The pots were covered with a shade cloth to prevent drying of the surface during germination. After one week, the shade cloth was removed and density was reduced to three plants per pot. Plants were watered as needed, usually every two days, with approximately 200 ml of deionized water to maintain field capacity. Pot locations were haphazardly adjusted weekly to insure equal light exposure in the greenhouse. Photosynthetically active photon flux density (PPFD 400-700 nm) in the greenhouse at solar noon, October 22, 1993, was 562 ± 135 µmol/m^2/s (mean ± SD) or 36% of outside PPFD that was 1542 ± 18 µmol/m^2/s. Light intensity was measured with a Li-Cor ® Li-1000 Data Logger with an integrating quantum sensor.

After fourteen weeks, plants were harvested and dry mass and percent infection measured. The above-ground shoots were removed by cutting at the soil surface, oven dried at 90° C for 48 hr and dry mass measured per pot. Roots were washed with tap water to remove soil. Wet root weights were recorded and a small root sample was obtained from three areas of the root for a total of approximately 0.3 g of sample. These samples were stained to determine percent mycorrhizal infection. The remaining roots were dried at 90° C for one week, and their dry mass measured. Ash-free root dry mass, minus the sample for staining, was obtained by ashing the dried roots at 700° C for 3 hr (Bohm 1979). The ash-free dry mass of the stained root samples was calculated by using a regression analysis of the ash-free dry mass versus final wet root weight. This gave the equation of a line which was used to estimate the ash-free dry mass of the sample from its wet weight. The equation for the line was $y = 0.119x + 0.000$ ($R^2 = 0.96$, P=0.001). Total root dry mass was determined by summing the ash-free dry mass with the estimated dry mass of the root sample used for staining.

Root samples in all experiments were stained for mycorrhizal detection using the trypan blue technique (Phillips and Hayman 1970). Fresh root samples were washed, cut into 1 cm segments, placed in 10% potassium hydroxide and heated for twenty minutes at 90°C. After clearing, root samples were rinsed, then placed in a mild bleach to further clear the roots. The bleach was removed and the root samples soaked in acidified water for ten minutes. After water removal, samples were stained in 0.05% trypan blue, de-stained and stored in 85% lactic acid until they were examined.

Root infections were determined using the modified gridline method (Newman 1966; Giovannetti and Mosse 1980; Van Auken and Brown 1998; Van Auken and Freidrich 2006). The lid from a 9.5 cm petri dish was used with gridlines at 0.5 cm intervals. Points where a root intersected a line at nearly a perpendicular angle were examined and only the area of the root over the line was considered when determining infection. The microscope was focused through the entire root and if any arbuscules or vesicles (but not coils or hyphae) were found, the root segment was considered infected. One hundred and fifty root-gridline intersections were examined for each sample and the percent infection calculated.

Aristida purpurea biomass and infection were determined in response to benomyl application in heat sterilized and non-sterilized soil. This was done to demonstrate that *A. purpurea* growth in sterile soil resulted from the mycorrhizal fungi being rendered inviable as opposed to the plant growth being a consequence of changes in other soil factors following the autoclaving procedure. To do so, *A. purpurea* plants were grown in sterile and unsterile soil in the presence and absence of the fungicide benomyl.

Ten 15 cm diameter by 15 cm deep pots were filled with 1400 g of dried non-sterile Patrick-series soil while another ten pots were filled with the same mass of soil heat sterilized by autoclaving. Five pots of each treatment were selected and a 100 ml suspension of benomyl was thoroughly mixed with soil in the pots. The benomyl concentration was 45 mg/kg in the soil. Benomyl is insoluble in water and was added to the soil as a suspension then thoroughly mixed.

Ten seeds of *A. purpurea* were sown per pot on July 27, 1994. The density was reduced to three plants per pot after one week. During the sixth week of growth, another 100 ml of a benomyl

suspension was added to the surface of the pots bringing the total concentration of benomyl to 90 mg/kg of soil. After 17 weeks, all plants were harvested on November 30, 1994. Shoot dry mass and ash-free root dry mass were measured along with percent infection of the roots. All dry mass measures were square root transformed and the percent infection was arc sine transformed to reduce variance (Lindman 1992). An F-test was used and is robust when the assumption of equal variances is violated as long as the samples are equal (Lindman 1992). SAS was used to conduct a two-way analysis of variance on shoot, root and total dry mass as well as percent infection with soil sterilization and benomyl treatment as main effects along with the interaction of these factors.

Non-nutrient agar was added to some pots to separate soil treatments. Biomass and infection of *A. purpurea* was examined in response to the addition of non-nutritive agar in sterile and non-sterile soil to demonstrate that it did not stimulate plant growth. Seven treatments were prepared in which sieved Patrick-series soil was mixed with increasing amounts of agar. The treatments were 0.0 g, 0.28 g, 0.56 g, 0.84 g, 1.13 g, 1.69 g and 2.25 g of undissolved agar mixed with 3400 g of soil and then added to 10.2 cm diameter x 40 cm tall pots constructed from PVC pipe. There were three replicates per treatment and care was taken to randomize soil/agar additions across treatments. Seeds were sown on March 9, 1994 and plants were harvested after a 14-week growing period. Shoot dry mass, root ash-free dry mass and percent mycorrhizal infection were measured. SAS was used for a one-way analysis of variance of response variables. Scheffe's multiple comparison tests were used to compare treatment means.

The response of *A. purpurea* to non-sterilized and heat sterilized soil as well as three non-sterile/sterile and three sterile/non-sterile stratified soil treatments was next examined. Shoot dry mass, root dry mass, along with total dry mass, and percent infection of the roots, every 10 cm in soil depth, was examined to describe the pattern of mycorrhizal infection and allocation of biomass throughout the vertical rhizosphere. Forty total pots, 10.2 cm diameter x 40 cm tall, were used, replication was 5 pots/treatment. Each pot was filled with 3400 g total of Patrick-series soil that was non-sterilized or heat sterilized. Some pots had different layers of soil stratified as non-sterile on top and sterile on the bottom (+/-), or the reverse (-/+). Various layers were prevented from mixing by placing a 1 cm thick layer of sterile, non-nutritive, agar (75 ml of a 7.5 g per 100 ml distilled H_20 mixture) between them. Each layer contained 850 g of the desired soil (four total) and was added with care taken to randomize the soil treatments. Pots were divided into four vertical segments of 10 cm each. After addition and solidification of the agar layer, the next layer of soil was added to fill the pot to its total of 3400 g. Five pots were setup for each of the following treatments: 40 cm non-sterile (+), 40 cm sterile (-), 20 cm-/20 cm+, 20 cm+/20 cm-, 30 cm-/10 cm+, 30 cm+/10 cm-, 10 cm-/30 cm+, and 10 cm+/30 cm-.

On July 29, 1993 *A. purpurea* seeds were sown as previously described. Plants were allowed to grow for 14 weeks and were harvested during a five day period from November 18, to November 22, 1993. One replicate per treatment was harvested each day. Roots were removed from the pots by flooding the pot with water, then inverting the pot to allow the soil and roots to slide out as a column. The column was then cut into 10 cm segments and the roots washed. A one-way analysis of variance was conducted to determine if the soil treatments affected the dry mass of the plants and the percent fungal infection. All response variables were transformed as previously indicated. Scheffe's multiple comparison test was used to determine significant differences because of its general conservatism.

The next experiment examined the biomass and root infection of *A. purpurea* in the presence of mycorrhizal fungi, soil stratification and the presence of a potential fungal propagule vector *Solenopsis invicta*, the red imported fire ant. Response variables were measured every 10 cm in soil depth. Sieved Patrick-series soil was non-sterile or sterile and placed in 10.2 cm diameter x 40 cm tall pots. Treatments consisted of five pots with all sterile (-) and five with all unsterile soil (+). Stratified treatments were set up with sterilized soil in the top 20 cm and non-sterilized soil in the bottom 20 cm of soil (20-/20+) as well as the reverse (20+/20-). A 1 cm thick agar layer was placed in each pot at a depth of 20 cm to prevent soil mixing. The same was done to the pots with all sterile

and all unsterile soil. Each treatment had five replicates. The four soil treatments were duplicated to examine potential effects of *S. invicta*. Thus, a total of forty pots were used, twenty with ants and twenty pots without ants.

Ten *Aristida purpurea* seeds were sown and thinned as previously noted. *Solenopsis invicta* were collected from a single colony located on the University of Texas at San Antonio campus. The mass equivalent of one thousand fire ant workers from a variety of castes was added to each of the ant treatment pots on April 17, 1994, three-and-half weeks after initial seed germination. At the same time, two queens and two hundred larvae, at various stages of development, from the same colony were added to these pots. The mean mass of one thousand S. *invicta* was 0.153 ± 0.012 g. This meant that on average each pot received 1000 ±80 ants.

Pots were modified to contain the *S. invicta* using thin, clear plastic, circular trays, 15.25 cm in diameter, with a circular hole cut in the center equal to the diameter of the pot. The tray was glued to the outside of the pot with silicone caulk. The inside wall of the tray was treated with a layer of fluon which prevented the ants from escaping. As the grass grew, some leaves were prostrate over the side of the tray, allowing the ants to escape. A cylinder, 9 cm in diameter by 7 cm tall, was made from 1.27 cm hardware cloth and placed on the soil surface. Pots without ants received the same modifications. The *S. invicta* ants were given water and a diet of a mixture of eggs, cooked hamburger, gelatin, sugar, salt, and a drop of vitamins (Banks 1981). Throughout the experiment, ant mortality was measured weekly by removing and counting the number of dead ants.

After 16 weeks, the live *S. invicta* adults and larvae were removed from the pots and the plants harvested over an eight-day period from July 2, to July 11. To remove the ants, a small hole was drilled in the bottom of the pot allowing it to be filled with water from the bottom to the top. Water was slowly added and the ants and larvae that were brought to the top were counted and removed. The shoots of the grass were cut at the soil surface and their dry mass measured. Root ash-free dry mass and percent infection was measured every 10 cm of pot depth.

A one-way analysis of variance was conducted on total ant mortality at the end of the experiment, total number of ants at harvest, the sum of total mortality and number of ants at harvest, and total number of larvae at harvest. Scheffe's multiple comparison test was used to separate treatment means. Transformations were as previously indicated. A two-way analysis of variance was performed with soil and *S. invicta* treatments as main effects along with their interaction. Student-Newman-Keuls multiple comparison test was used to separate treatment response variables (Einot and Gabriel 1975).

RESULTS

Biomass and percent root infection of *Aristida purpurea* were significantly reduced by soil heat sterilization and benomyl treatment (two-way ANOVA, $P < 0.0001$ for all). In non-sterile soil, mean total plant dry mass without benomyl was 3.01 g and with benomyl it was 0.05 g. Mean percent infection in non-sterile soil without benomyl was 27.5 % and with benomyl it was 2.0 %. Mean total *A. purpurea* dry mass in heat sterile soil was 0.06 g without benomyl and 0.04 g with benomyl, while the mean percent infection in heat sterile soil was 0.0 % with or without benomyl. Trends for shoot and root dry mass were the same as for total dry mass.

Addition of non-nutrient agar did not significantly affect dry mass or percent infection of *A. purpurea* in non-heat sterilized soil (one-way ANOVA, $P > 0.05$ for all, Figure 1). Percent infection ranged from 28.7 % to 43.6 % and was not significantly different. Shoot dry mass was approximately twice as high as the root

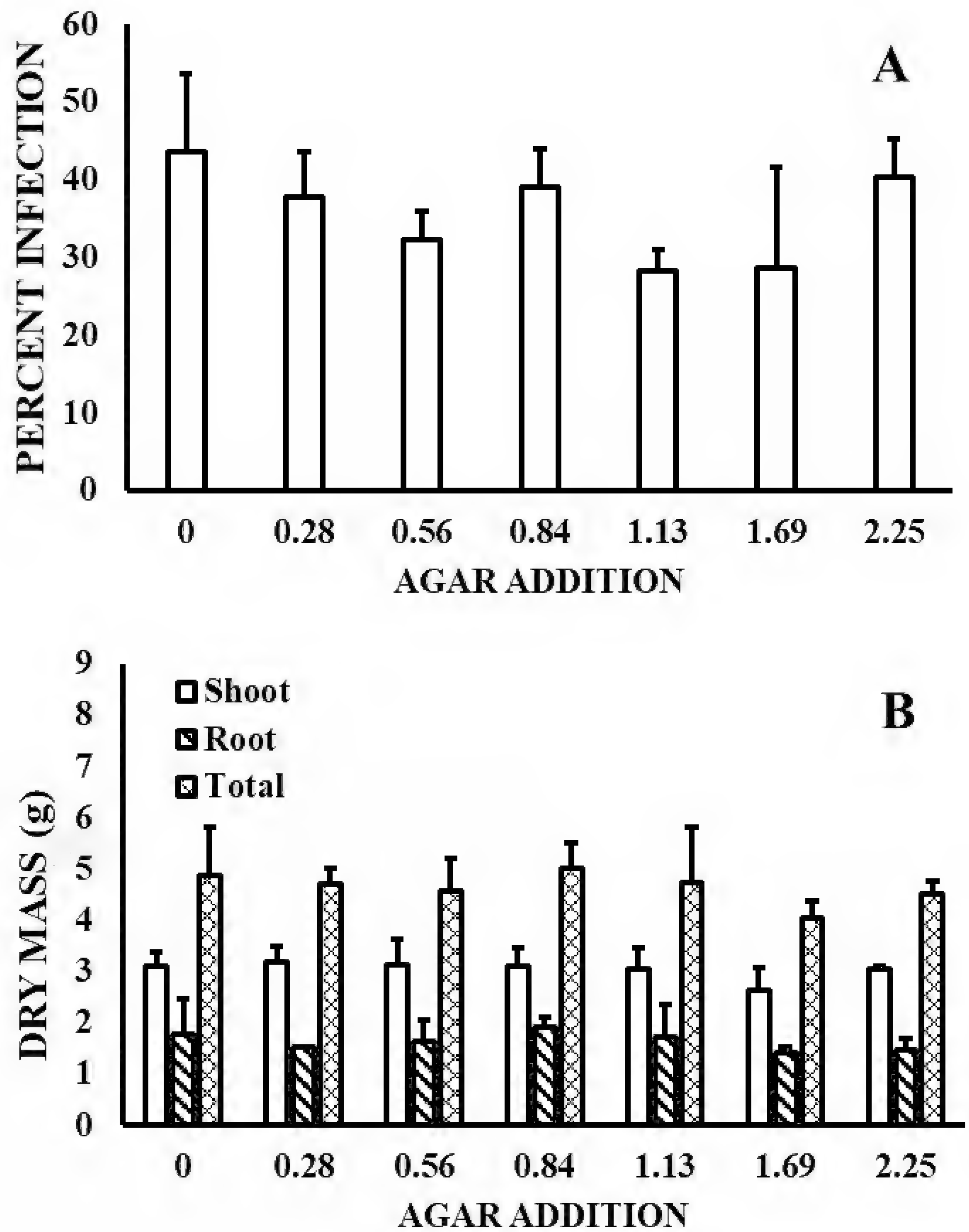

Figure 1. Mean percent infection (A) and mean root, shoot and total dry mass (B) of *Aristida purpurea* for each non-nutrient agar addition in grams in non-sterile soil. The line at the top of each bar is + one standard deviation of the mean.

dry mass and total dry mass ranged from 4.04 to 5.01 g/pot. The effects of addition of non-nutritive agar on the dry mass and percent infection of *A. purpurea* in heat sterilized soil showed that shoot dry mass was significantly affected by agar addition ($P < 0.05$), but values and differences were small with high variability, and the root and total dry mass values were not significantly affected by agar addition ($P > 0.05$ for both, not shown). Percent root infection for all was zero.

Overall results of the effects of various soil stratifications on the response variables of *A. purpurea* showed that shoot, root, total dry mass and percent root infection measurements were all significantly affected by the various soil stratifications (one-way ANOVA, $P < 0.0001$ for all). Based on Scheffe's multiple comparison test, *A. purpurea* response variables were all significantly reduced in plants grown in sterile soil (all-, $P < 0.05$) compared to those in the non-sterile control (all+). Mean shoot, root and total dry mass for plants grown in sterile soil were 0.04 g, 0.04 g, and 0.08 g,

respectively (Table1). In non-sterile soil, mean dry masses were 1.82 g, 2.52 g and 4.34 g, respectively (Table 1). Mean percent infection per pot was reduced from 13.1 % in the non-sterile soil control to 1.7 % in sterile soil (Table 1).

Table 1. Mean shoot, root, total dry mass and percent infection of *A. purpurea* for each soil treatment. A – indicates the soil was sterilized while a + indicates non-sterile. Dry mass means are presented in grams. Values for a treatment in a column with the same letter are not significantly different (Scheffe's multiple comparison test, P > 0.05).

Soil Treatment	MEAN DRY MASS (g)			% Root Infection
	Shoot	Root	Total	
ALL -	0.04b	0.04c	0.08c	1.7c
ALL +	1.82a	2.52a	4.34a	13.1a
20+/20-	2.46a	3.63a	6.09ab	11.4ab
20-/20+	1.70a	2.51a	4.21ab	6.3abc
30+/10-	2.47a	3.28a	5.76ab	13.9a
30-/10+	0.14b	0.23b	0.37c	5.1bc
10+/30-	2.06a	3.72a	5.78ab	13.0a
10-/30+	2.47a	4.28a	6.75a	12.9a

The Scheffe multiple comparison test revealed that none of the stratified soil treatment significantly increased mean total dry mass above that of the non-sterile control (all+, $P > 0.05$, Table 1). Mean total dry mass in the 10-/30+ treatment was 6.75 g compared to 4.34 g for the non-sterile control, but not significantly different (Table 1). In all soil treatments, except the sterile treatment (all- and the 30-/10+), mean root dry mass exceeded mean shoot dry mass from 1.33 to 3.1 times (Table 1).

Vertical soil profiles of the rhizosphere showed where the root was infected and how that modified dry mass. For *A. purpurea* plants grown in sterile soil (all-) the vertical pattern of infection was low compared to plants in non-sterile soil (all+, Figure 2A). In the non-sterile (all+) soil, infection decreased gradually and uniformly from 18.3% in the top 10 cm section to 13.6% in the 30 cm depth followed by a rapid decline to 4.9% in the 40 cm bottom section (Figure 2A). Mean percent infection for the entire pot was 13.1%. In the 20+/20- soil treatment, the pattern of infection was similar to the non-sterile treatment. However, percent infection in the 20 cm section and the 30 cm section (Figure 2A) were equal but the 20 cm section of root was in the non-sterile soil while the 30 cm section was in sterile soil. Infection in the 40 cm segment was 2.9%. For plants in the 20-/20+ treatment, infection was reduced but highest in the 30 cm section of the pot at 10.5% (Figure 2A). The roots in the 10 cm and 20 cm sections (in sterile soil) were equally infected but higher than those in all segments of the all sterile treatment (Figure 2A).

For plants in the 30+/10- treatment, the vertical pattern of infection was similar to that in non-sterile soil (all+), except that there was an increase in percent infection from the 30 cm section (unsterile soil) to the 40 cm section (sterile soil, Figure 2B). In the 30-/10+ treatment, percent infection was generally lower, but increased in the 40 cm section with high variance (Figure 2B). The pattern of infection in the 10+/30- treatment was asymmetrical in that the 10 and 20 cm root sections were much

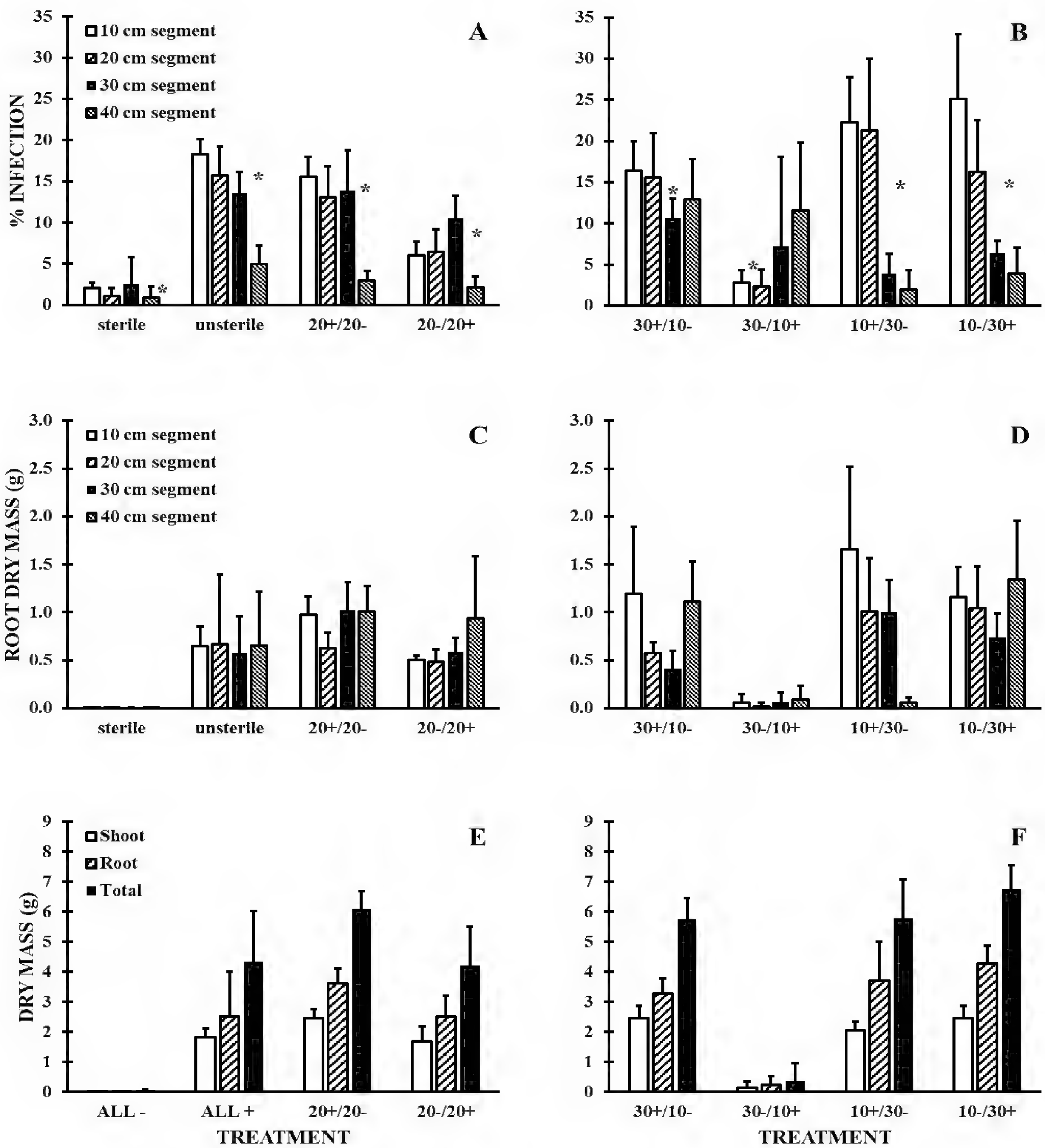

Figure 2. Mean percent infection (A, B), root dry mass for each 10 cm pot segment (C, D), and mean total shoot, root, and total dry mass (E, F) for *Aristida purpurea* in each soil treatment. Mean per pot infection = (*) and line at top of bar is +1 SD. A – is sterile and + is non-sterile soil.

more highly infected than the 30 and 40 cm sections (~21.0% vs. ~3.0%, Figure 2B). The 10-/30+ treatment was similar to the previous treatment but showed a more progressive decrease in percent infection from 25.1 % in the 10 cm root section to 3.9 % in the 40 cm section (Figure 2B). Percent infection in the -10 cm root section was highest among all root sections from every treatment and occurred in a region of sterile soil (Figure 2B).

Allocation of root dry mass throughout the vertical rhizosphere in sterile soil (all-) was very low (Figure 2C). In non-sterile soil (all+) root dry mass was similar in each section. In the 20+/20- soil treatment, root dry mass was higher but allocated almost equally in all root sections (Figure 2C). For plants in the 20-/20+ treatment, root dry mass increased slightly with depth in the non-sterile soil (Figure 2C). Root dry mass in the 30+/10- soil treatment, was higher in the 10 cm and 40 cm root

sections when compared with the 20 cm and 30 cm sections (Figure 2D). Root dry mass in the 30-/10+ treatment, was quite low in each section (Figure 2D). In the 10+/30- soil treatment, root dry mass in the 10 cm section was highest and then dropped with increasing depth and there was very little root dry mass in the deepest soil segment that was sterile (Figure 2D). For plants in the 10-/30+ treatment, root dry mass dropped from the 10 to the 30 cm section but increased in the 40 cm non-sterile soil section (Figure 2D). Total dry mass was low in the all- segments of the sterile soil treatment (Figure 2E). Total dry mass was higher in all treatments except the 30-/10+ and was highest in the 10-/30+ soil treatment.

Solenopsis invicta was examined as a potential fungal vector in pots with *A. purpurea.* Total mortality of *S. invicta* differed significantly with soil treatment (one-way ANOVA, $P < 0.05$, Figure 3). However, the number of larvae at harvest, the number of adults at harvest and the sum of live and dead *S. invicta* at harvest were not significantly different in the soil treatments (one-way ANOVA, $P > 0.05$, Figure 3A, C and D), but total mortality was significantly different (one-way ANOVA, $P < 0.05$, Figure 3B). Mean total *S. invicta* mortality was significantly lower in the non-sterile (all+) and 20+/20- soil treatments compared to the sterile treatment (all-) but not significantly different in the 20-/20+ treatment (Scheffe multiple comparison test, $P > 0.05$). Mean total mortality in the sterile soil treatment was 477.6 and 380.6 in the 20-/20+ treatment, while it was 237.4 in the non-sterile treatment and 202.4 in the 20+/20- treatment. Even though approximately one thousand *S. invicta* adults were added to each pot initially, at the end of the experiment mean sum of total ants including mortalities was 583-887 ants for all soil treatments with no significant differences.

Overall, *Aristida purpurea* shoot and total dry mass were significantly affected by the presence of *S. invicta* (two-way ANOVA, $P < 0.05$). However, root dry mass and percent infection of *A. purpurea* were not significantly affected by *S. invicta* (two-way ANOVA, $P > 0.05$). Soil treatment had a significant effect on *A. purpurea* response variables (two-way ANOVA, $P < 0.0001$). The two-way interaction of *S. invicta* and soil treatment was significant only for shoot dry mass meaning that *A. purpurea* shoot dry mass increased significantly with *S. invicta* but only in non-sterile soil (two-way ANOVA, $P < 0.05$). Student-Newman-Keuls groupings of shoot, root and total dry mass were significantly lower in the sterile (all-) and 20-/20+ soil treatments compared to the non-sterile (all+) and 20+/20- soil treatments (Table 2). Also, the percent infection per pot was significantly lower in sterile soil (all-) compared to the 20-/20+ soil treatment (Table 2). Percent infection in the 20-/20+ soil treatment was significantly lower than that in the non-sterile (all+) and 20+/20- soil treatments (Table 2, Figure 4). The only significant difference in shoot and total *A. purpurea* dry mass in the presence of *S. invicta* occurred in non-sterile soil (all+) where shoot and total dry mass increased by 67.3% and 49.3%, respectively (Table 2, Figure 4). With *S. invicta* present in the 20+/20- soil treatment, shoot and total dry mass increased by 19.0% and 16.3%, respectively, but not significantly (Table 2). In the sterile (all-) and 20-/20+ soil treatments, shoot and total *A. purpurea* dry mass decreased with *S. invicta* compared to without *S. invicta*, but was not significantly different (Table 2). It is also interesting to note that without *S. invicta* present, Student- Newman-Keuls groupings show that shoot and total dry mass of *A. purpurea* were significantly higher in the 20+/20- soil treatment compared to the unsterile soil treatment even though percent infection was lower for the 20+/20- treatment, but not significantly lower.

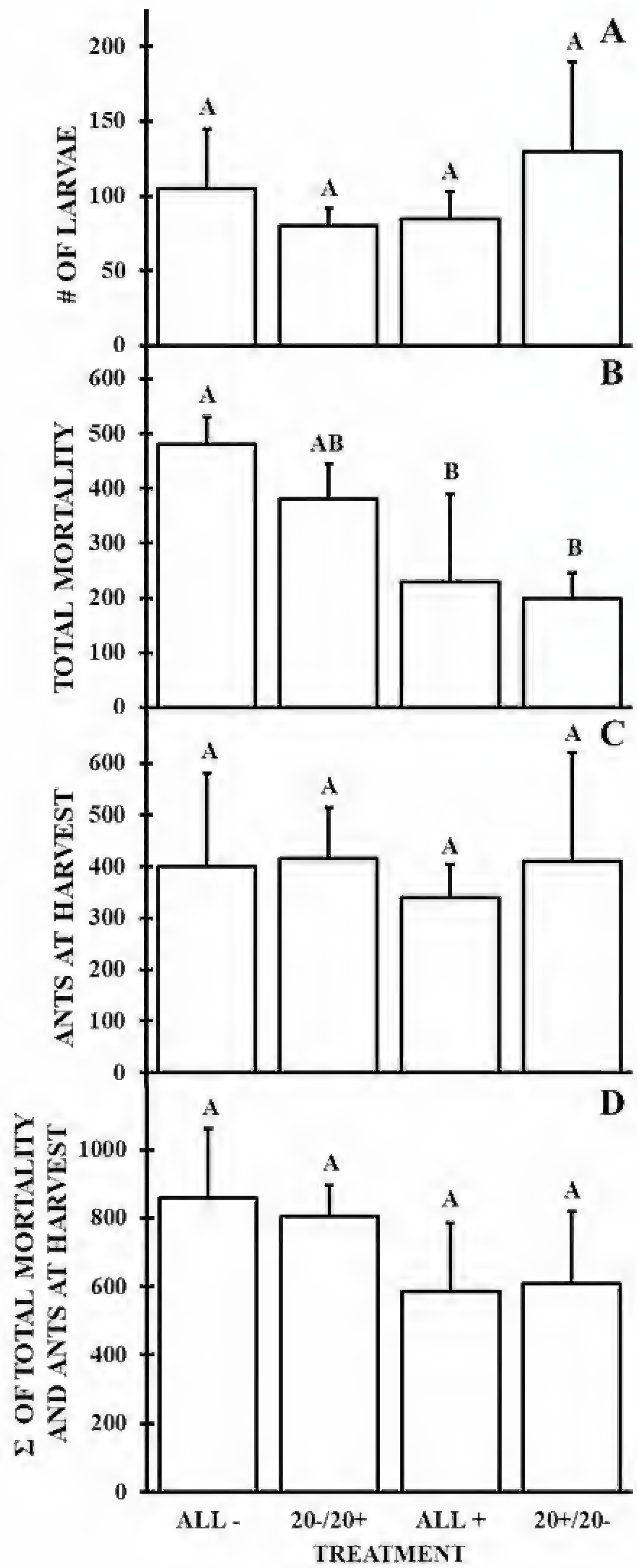

Figure 3. Mean number of larva (A), total mortality (B), adult ants at harvest (C), and sum of total mortality and adult ants at harvest (D). Line at the top of a bar is 1 SD. Means with the same letter at the top are not significantly different (Scheffe's multiple comparison).

Table 2. Mean shoot, root, and total dry mass and mean percent infection per pot of *A. purpurea* for each soil and ant treatment. Means for a treatment in a column with the same letter possess means which are not significantly different when tested with Student-Newman-Keuls multiple comparison test. A - indicates sterile soil while + indicates unsterile soil.

		MEAN DRY MASS (g)			
Soil Treatment	Ant Treatment	Shoot	Root	Total	% Root Infection
ALL -	w/ ants	0.03c	0.02c	0.05c	0.22c
	w/o ants	0.04c	0.02c	0.06c	0.22c
20-/20+	w/ ants	0.12c	0.10c	0.22c	4.6b
	w/o ants	0.19c	0.18c	0.37c	7.5b
ALL+	w/ ants	2.81a	2.7ab	5.51a	35.0a
	w/o ants	1.68b	2.03b	3.71b	40.8a
20+/20-	w/ ants	2.94a	3.13a	6.07a	26.4a
	w/o ants	2.47a	2.76ab	5.23a	26.5a

Examination of the pattern of percent mycorrhizal infection throughout the vertical rhizosphere showed that *A. purpurea* plants grown in sterile (all-) soil, with and without *S. invicta,* had very low levels of root infection ranging from 0.0% to 4.5% for all root sections (Figure 5A). In the 20-/20+ soil treatments with *S. invicta,* percent infections were low and somewhat uniform throughout the root ranging from 3.3% in the 30 cm section to 8.0% in the 20 cm section (Figure 5A). Without *S. invicta,* percent infection increased to 16.2% and 13.8% in the 20 cm and 30 cm (heat sterilized) root sections although variation was high (±28.1% and ±16.9% respectively, Figure 5A). The non-sterile (all+) soil treatment was high, with and without *S invicta*, and showed a similar decrease in percent infection with increasing soil depth from about 70.0% to about 15.0% (Figure 5B). In the 20+/20- soil treatment, with and without *S. invicta*, percent infection decreased as soil depth increased with little variation between the two treatments. Regardless of ant treatment, percent infection for the 40 cm root segment in the 20+/20- soil treatment, ranged from 0.0% to 0.3% and was much lower than for the non-sterile treatment which ranged from 11.0% to 15.1 % (Figure 5B). There was little root dry mass in all soil sections in the vertical rhizosphere of *A. purpurea* plants grown in sterile soil (all- or 20-/20+) with or without *S. invicta* (Figure 5C). For *A. purpurea* plants in the non-sterile soil treatment (All+, 20+/20-), root dry mass allocation increased slightly with depth with and without *S. invicta*. In the 20+/20- soil treatment, root dry mass allocation was more uniform with *S. invicta* than without them (Figure 5D). All dry mass measured in the sterile soil treatments were very low (Figure 5E).

Total dry mass for the all+ treatments were significantly different with less dry mass when *S. invicta* was absent. In the 20+/20- total *A. purpurea* dry mass there were no significant difference with or without *S. invicta* but total dry mass was higher when ants were present (Figure 5F).

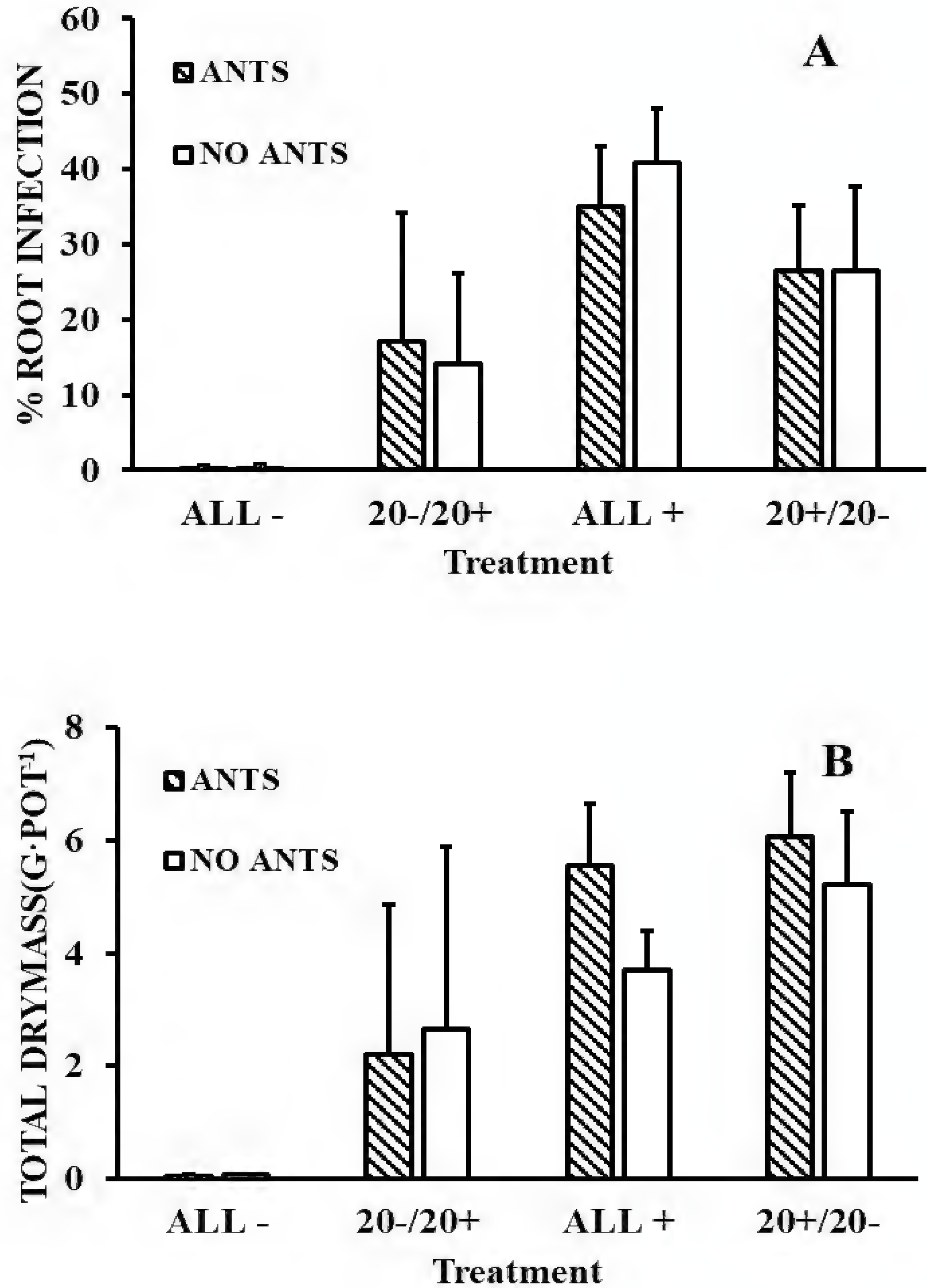

Figure 4. Mean percent infection per pot (A) and mean total dry mass per pot of *Aristida purpurea* for each soil and *Solenopsis invicta* treatment. The line at the top of a bar is + 1 SD. A – indicates sterile soil while a + is non-sterile.

DISCUSSION

Soils are complex and effects of abiotic and biotic soil factors on higher plants are difficult to sort out (Willis et al. 2013; Wills and Landis 2018). Of the biotic factors in the soils, arbuscular mycorrhizal fungi are particularly important to the growth and survival of most vascular plants. Over 80% of terrestrial plants are infected with AM fungi with 95% of terrestrial plant families having species with mycorrhizal associations including the Poaceae or grasses (Trappe 1987; Wang and Qui 2006; Bush 2008). This mutualistic association includes the fungal contribution to the higher plants' mineral nutrition especially the uptake of phosphorus and other limiting soil nutrients and the plant contributing carbohydrates to the fungus (Bolan 1991; Clark and Zeto 2000; Bush 2008; Leigh et al. 2008; Willis et al. 2013). Mycorrhizae are well-known to increase plant growth especially in nutrient poor soils (Gianinazzi 1991; Van Auken and Brown 1998; Van Auken and Fredrick 2006; Bush 2008; Willis et al. 2013; Keddy 2017). But, there are other ways that these fungi can alter the organization and even structure of various plant communities (Bush 2008; van der Heijden et al. 2008; Willis et al. 2013; Wills and Landis 2018).

Grassland plants such as *Aristida purpurea* and grassland or prairie ecosystems have been and should continue to be a focus of research on mycorrhizal associations. Grasslands support a high degree of diversity of both plants and animals (Wills and Landis 2018). In addition, over 90% of North American grasslands have been converted to other uses, mainly agriculture (Samson and Knopf 1994). *Aristida purpurea* is a C_4 warm season grass that seems to be early successional or a grass that uses or exploits disturbances (Gould 1975; Van Auken and Brown 1998). It is found at low to mid-elevations across the central United States to the Pacific Ocean, in the western provinces of Canada, and the northern states of Mexico (USDA-NRCS 2019). This species is one of many C_4 grasses that grow in disturbances caused by a number of different influences including heavy cattle grazing. The colonization of the roots of these grasses by mycorrhizal spores in the soil is necessary for their growth and could be assisted by various soil organisms including one or more native or introduced Hymenoptera, specifically some of the Formicidae: ants (Wills and Landis 2018). The role of ants in grasslands is important but not completely understood. They are considered ecosystem engineers and can influence the population size and diversity of various invertebrates, plants and soil microorganisms (Holldobler and Wilson 1990; Del Toro et al. 2012; Boulton and Amberman 2006; Sanders and van Veen 2011; Wills and Landis 2018).

We have confirmed that heat sterilized soil reduced *A. purpurea* growth to almost zero. Also, the addition of the fungicide benomyl to soil reduced growth of *A. purpurea* to almost zero, similar to a previous report (Van Auken and Brown 1998). This demonstrated that the cause of the reduction in dry mass was due to the reduction or elimination of viable mycorrhizal propagules by the fungicide and not as a consequence of other potential changes to the soil caused by temperature. Further evidence showed the fungicide benomyl caused the reduction of the percent infection of *A. purpurea* roots in benomyl treatment soil. The effect of the fungicide on the growth of *A. purpurea* was analogous to that of soil heat sterilization. Thus, fungicide or heat sterilization could be used to eliminate soil fungi, but the reduction of other soil organisms by heat sterilization did not affect *A. purpurea* dry mass production or growth. Objections raised by others that killing all other soil organisms including the fungi, would cause potential masking of mycorrhizal effects are not warranted (Stribley 1987). Neither mortality nor number of seeds produced was assessed in this experiment, but all plants grown in sterile soil survived but never produced seed, while those grown in unsterile soil produced seeds (Engelken 1995).

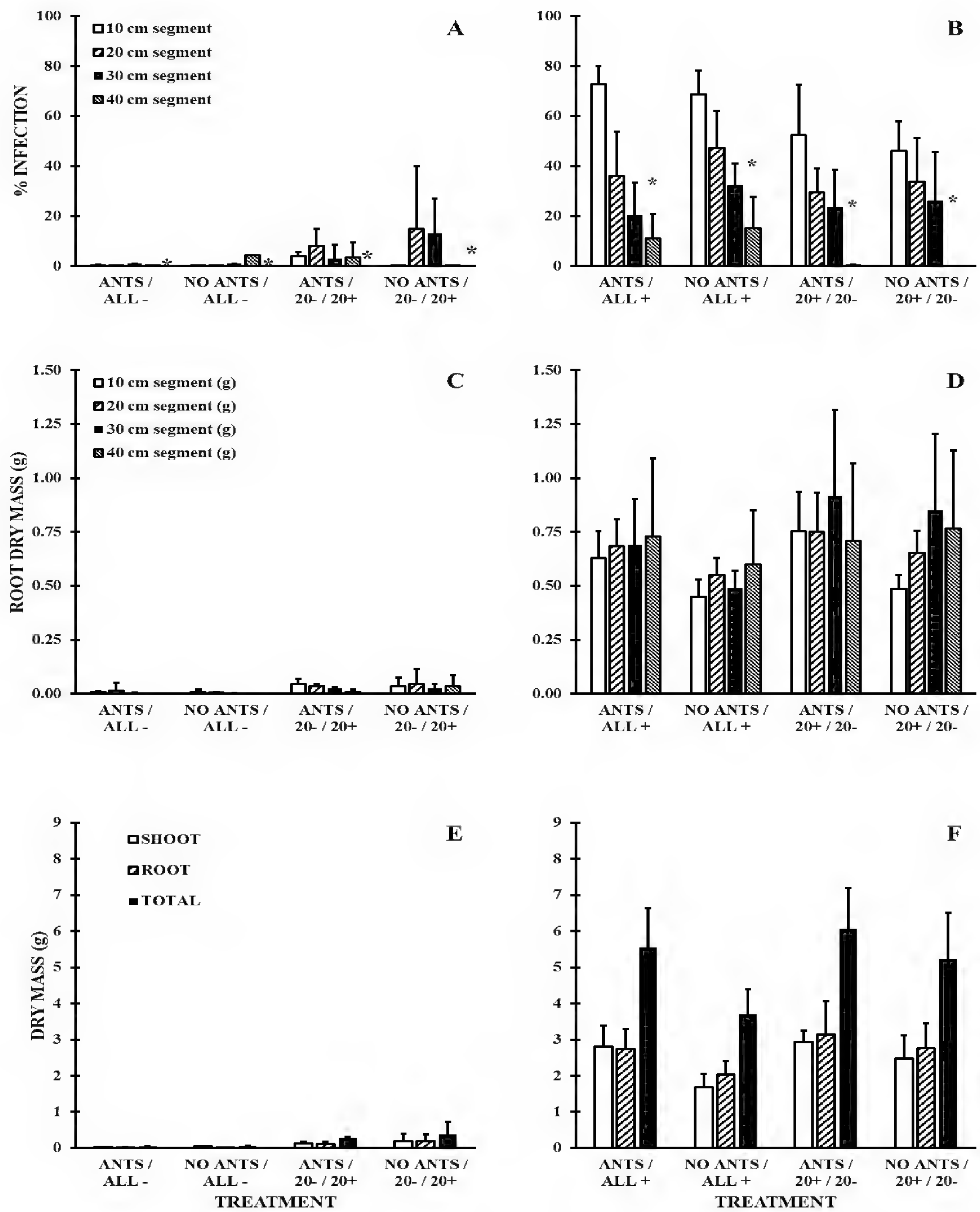

Figure 5. Mean *A. purpurea* percent infection (A, B) and root dry mass (C, D) per 10 cm pot section and total shoot, root and pot dry mass for each soil and *S. invicta* treatment (E,F). The line at the top of a bar is + 1 SD. Sterile soil is indicated by a - while non-sterile is indicated by a +.

Because *A. purpurea* grows in disturbances, we wanted to determine how much of a disturbance to the soil they could survive. Before we could do that, we had to determine how to spatially separate sterile (disturbed) and non-sterile (non-disturbed) soil, thus another experiment was required. We added increasing amounts of non-nutrient agar to pots with non-sterile soil and *A. purpurea* to see if root percent infection decreased and if plant dry mass increased due to a fertilizer effect by the addition of the agar (Willis et al. 2013). The addition of non-nutritive agar to the soil did not act as a fertilizer. For agar addition in sterile soil, there was a slight but significant increase in *A. purpurea* shoot dry mass, but this increase was so small as to be biologically unimportant and

potentially not an effect of the agar but an artifact. Generally, fertilizer addition results in a significant reduction in mycorrhizal infection because greater nutrient availability in the soil renders the mycorrhizal symbiosis superfluous (Menge et al. 1978). Therefore, if agar addition acted as an effective fertilizer, mycorrhizal infection would have been lower with increasing agar addition. This would also be coupled to increases in plant dry mass as reported by others (Owusu-Bennoah and Mosse 1979; Hijri et al. 2006; Willis et al. 2013). The results of the current study demonstrated that neither of these were the case.

When the upper layer of soil, top 0-30 cm, is severely disturbed, the mycorrhizal inoculum potential is considerably reduced (Jasper et al. 1979; Willis et al. 2013). The reduction could be short term, depending on degree of disturbance and local environmental conditions. Consequently, we examined the root infection and ability of *A. purpurea* to grow through a sterile layer of soil. Because *A. purpurea* is a species found in disturbances, it would have to start growth in disturbed surface soil. We examined the effect of various sterile/non-sterile soil stratifications on the growth and percent infection of *A. purpurea.* We showed that if the upper 20 cm of pot soil was sterile that root infection was reduced by approximately 83% and total dry mass was reduced by approximately 90%. In addition, results showed that when mycorrhizal fungi infected the roots of *A. purpurea*, and the roots grew into sterile soil, there was an increase in dry mass of the plant. The increase was mostly in the shoot. Increases in shoot:root ratios of mycorrhizal plants have been attributed to enhanced efficacy of the mycorrhizal association present (Marschner and Dell 1994). However, some studies with C_4 grasses have shown that soil microflora can negatively affect the mycorrhizal symbiosis (Daniels Hetrick et al.1988; Hetrick et al.1988), possibly by competing for nutrients, resulting in reduced plant dry mass and root infection. It appears that *A. purpurea* plants with roots that are infected with mycorrhizae in sterile soil avoid the antagonistic effects of other microorganisms and are more effective taking up nutrients than those plants grown in non-sterile soil. The roots in sterile soil may have become infected as external hyphae grow and extend along the length of the root from primary infection sites in non-sterile soil to secondary sites in sterile soil (Sanders and Sheikh 1983).

When looking at the pattern of mycorrhizal infection of *A. purpurea* roots in the vertical rhizosphere, percent infection decreased with increasing soil depth in unsterile soil even though propagule density was initially uniform throughout. This pattern of infection was seen with grasses in field experiments by others (Zajicek et al. 1986). It is also interesting to note even though the mixed non-sterile and sterile, soil treatments had a different pattern of infection, the percent root infection per pot of these treatments had a very narrow range 12.9% to 13.9%. It appears that mycorrhizae formation in the roots of *A. purpurea* may be under internal control whereby further root infection would be inhibited once the percent infection reaches an optimum level. This could be an advantage for the grass because further infection may not improve nutrient uptake above the cost of supporting the growth of the fungus. Research with ryegrass and sudan-grass showed that increased phosphate within the plant resulted in reduced percent mycorrhizae along with reduced soluble carbohydrates which may be a control mechanism (Menge et al. 1978; Jasper et al. 1979). Delaying initial mycorrhizal infection does not seem to enhance the growth of *A. purpurea* because when the upper layer of soil was sterile there was no significant difference from the non-sterile soil. However, if the upper layer of soil does not contain fungal propagules and exceeds 20 - 30 cm, the grass cannot produce enough dry mass to establish, grow, and survive, which has been reported for other species (Willis et al. 2013).

Soil generalist, such as many hymenopterans, may be important for the establishment and growth of *A. purpurea.* Species of ants like *S. invicta* the imported red fire ant have become dominant species in much of the southern United States. Despite claims of considerable environmental or ecosystem disservice, control of *S. invicta* seems to be lacking (Wills and Landis 2018). More attention should be paid to species like this, especially their belowground effects. Little is known about what to expect from introduced species in the future especially as the climate continues to change (Pagano and Gupta 2016; Wills and Landis 2018). Disturbance tolerant ants such as *S. invicta* are often

invasive and will most probably influence native species populations in a negative way (LeBrun et al. 2013; Moranz et al. 2013).

We examined the potential for *S. invicta* to be a vector of fungal propagules. *Solenopsis invicta* does not appear to act as a vector for the transport of fungal propagules which could establish mycorrhizal associations in the roots of *A. purpurea.* This is evidenced by the similar patterns of infection in the vertical rhizosphere of *A. purpurea* between treatments with and without ants. Perhaps *S. invicta* moving soil brought nutrients to the plant roots which in effect could replace the fungal hyphae (Wills and Landis 2018). Examination of the root dry mass allocation in the non-sterile and 20+/20- soil treatments, without *S. invicta* revealed that root dry mass increased primarily in the sterile soil regions of the pot. When *S. invicta* was present in the 20+/20- soil treatment, root dry mass appeared to increase only in the non-sterile soil regions but not in the sterile soil. However, in non-sterile soil only, root dry mass in each of the four 10 cm segments was higher in the presence of *S. invicta.* Also, the increase in *A. purpurea* total dry mass in the 20+/20- soil treatment with *S. invicta* compared to without the ant was almost one-half that seen in the non-sterile soil treatment.

The mechanism by which *S. invicta* could affect the growth of *A. purpurea* in non-sterile soil is not certain but seems likely to be at the level of the mycorrhizal symbiosis since changes in *A. purpurea* growth in the presence of *S. invicta* were greatest when roots were most highly infected and because *A. purpurea* is obligatorily dependent upon mycorrhizae for growth. However, looking at the results from across all experiments, the presence of mycorrhizal fungi in the roots of *A. purpurea* does not explain by itself the growth of the grass. When looking at *A. purpurea* plants that became infected with mycorrhizae across all experiments in time, there was not a significant linear correlation between percent infection and total *A. purpurea* dry mass (Pearson's correlation, Engelken 1995). This suggests that the relationship probably involves several factors that may not be easily identified or controlled. Plant growth and yield is optimized when nutrient uptake by the fungus and its delivery to the host plant is equivalent to, or exceeds, the carbohydrate demands of the fungus (Smith et al, 1994). It is possible that the presence of *S. invicta* may enhance the efficacy of the mycorrhizal symbiosis directly by reducing mycorrhizal fungal biomass through feeding on external hyphae (Lanza 1991). This reduction in fungal biomass may be sufficient to lower the requirements of the fungus imposed on the host without significantly diminishing nutrient uptake. Also, the venom of *S. invicta*, an alkaloid having antibacterial, antifungal and insecticidal activity could reduce other fungal biomass (Blum 1985). The dispersal of venom may also act indirectly by reducing the number of microorganisms in the soil (Bruno de Carvalho et al. 2019), which could act antagonistically toward the mycorrhizal symbiosis in a manner analogous to sterilization (Fitter and Garbaye 1994). Because significant effects on *A. purpurea* growth were not seen in all soil treatments, it is likely that other potential chemical, physical or biotic changes to the soil as a result of *S. invicta* activity could have been important factors influencing these findings.

Interpretation of these results and others are difficult due to the complexity of the interactions between *A. purpurea,* the mycorrhizal fungi, and the soil environment including *S. invicta*. Temporal effects such as timing of rainfall events or other environmental conditions could also be important when comparing experiments in time. Dry mass measures of *A. purpurae* were not noticeably changed over the two years of these experiments although percent infection did change (Engelken 1995). It seems that the effects of mycorrhizal symbiosis on the biomass of *A. purpurea* varies depending upon a number of factors that were not identified and appear difficult to control. The quantity of mycorrhizal infection in plant roots and the abundance of propagules in the soil can change throughout the season and timing of infection is likely to be important for plant growth (Abbott and Gazey 1994). Also, all mycorrhizal fungi do not contribute equally to nutrient uptake and plant growth and thus roots may be highly infected, but with a variety of fungi that have a wide range of effectiveness and responses to environmental conditions (Brundrett 1991; Wills and Landis 2018). Despite the difficulty in predicting the effects of unknown mixed factors on plant growth, it appears that *A. purpurea* is an obligate mycotroph dependent upon the fungi for its growth, but other soil factors including certain biota such

as *S. invicta* can potentially alter the effectiveness of the mycorrhizal symbiosis and consequently the growth of *A. purpurea.*

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